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PROPERTIES OF TONIC CONTRACTIONS PRODUCED BY ELECTRICAL STIMULATION OF THE ANTERIOR RETRACTOR OF THE BYSSUS OF *MYTILUS EDULIS*

NOTE OF CORRECTION

In the paper entitled "Changes in muscle contraction curves produced by drugs of the eserine and curarine groups", by **G. Briscoe** (*J. Physiol.* [1938], 93, 194), mention was made of two hordenine compounds which had been tested for their peripheral and central effects. These compounds were the dimethyl carbamic esters of hordenine hydrochloride and of hordenine methyl sulphate, not hordenine hydrochloride and hordenine methyl sulphate.

Schweitzer & Wright (*J. Physiol.* [1938], 92, 422) have shown that hordenine hydrochloride and hordenine methyl iodide have actions on the central nervous system quite different from that of the dimethyl carbamic compounds.

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paper an attempt has been made to show that tonic contractions produced as a result of stimulating the muscle with A.C. or D.C. are also due to the action of ions present outside the muscle fibres.

METHODS

The muscle was stimulated as described previously. An easy method for determining small changes in the rate of relaxation was used as follows. If the periods of stimulation with A.C. or D.C. (for 10 sec.) be made at such a frequency that the relaxation of the muscle between successive periods is incomplete, the base-line, as drawn through the beginning of each contraction, rises in an even slope. If the rate of relaxation decreases, the base-line rises more rapidly; if it increases, the base-line drops. A convenient frequency of stimulation with A.C. or D.C. for 10 sec. is once a minute.

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PROPERTIES OF TONIC CONTRACTIONS PRODUCED BY ELECTRICAL STIMULATION OF THE ANTERIOR RETRACTOR OF THE BYSSUS OF *MYTILUS EDULIS*

By INDERJIT SINGH

From the Physiological Laboratory, Cambridge

(*Received* 20 *April* 1938)

WINTON [1937] has described the tonic contraction of ***Mytilus*** muscle produced by stimulation with direct current (D.C.). Singh [1938*b*] has described the properties of tonic contractions produced by certain ions. In this paper are described the properties of tonic contractions produced as a result of stimulating the muscle with alternating current (A.C.) or D.C.

In previous papers [Singh, 1938*b*, *c*, *d*] it has been suggested that tonic contractions of high viscosity are due to action of ions present outside the muscle fibres, while the A.C. contraction, characterized by low viscosity, is due to action of ions present inside the muscle fibres. In this paper an attempt has been made to show that tonic contractions produced as a result of stimulating the muscle with A.C. or D.C. are also due to the action of ions present outside the muscle fibres.

METHODS

The muscle was stimulated as described previously. An easy method for determining small changes in the rate of relaxation was used as follows. If the periods of stimulation with A.C. or D.C. (for 10 sec.) be made at such a frequency that the relaxation of the muscle between successive periods is incomplete, the base-line, as drawn through the beginning of each contraction, rises in an even slope. If the rate of relaxation decreases, the base-line rises more rapidly; if it increases, the base-line drops. A convenient frequency of stimulation with A.C. or D.C. for 10 sec. is once a minute.

RESULTS

Properties of the A.C. *off-contracture*

This contracture is produced on cessation or sudden reduction of an A.C. stimulus [Singh, 1938*b*]. Usually the duration of the passage of the current to produce the contraction must exceed 2–3 min., though in potassium sensitive muscles, passage for 10 sec. may be sufficient. The tension produced may be as high as 60 g. Almost maximum response is obtained with passage of the current for 3–5 min., with further increase in duration up to 10–20 min., the response increases by 5–10 p.c. and thereafter it remains constant or slightly diminishes by 10–20 p.c. even if the current is continued for 2 hr. The response increases with the voltage employed; if the contracture is not obtained with 10 V., it may be obtained if the voltage is increased to 15, 20 or 24 V.

The response increases with increase of temperature up to 24–26° C., thereafter it diminishes. With increase of temperature up to 37° C., adaptation to A.C. decreases, so that the tension at the end of the period of stimulation is high, and the contracture has to be superimposed upon this tension. It is therefore difficult to say whether the diminution of the contracture after 26° C. is due to the increase of temperature *per se* or not; the combined tension produced on cessation of A.C. (10 V.-5 min.), however, rises up to 37° C., and then declines.

The A.C. off-contracture is interesting in view of the fact that while it is produced as a result of passage of A.C. its properties are those of the potassium contraction. Thus: (1) it is absent in muscles inexcitable to potassium (0·1 *M* KCl); (2) its relaxation is slow; (3) it is diminished during the staircase effect produced by frequent stimulation with A.C.; (4) it is increased during fatigue; (5) it is inhibited by calcium; (6) the optimum concentration of magnesium is the same as that for potassium (0·018 *M* $MgCl_2$), whereas the response to A.C. is depressed by magnesium in all concentrations; (7) the anions increase it in the order $NaCl < NaBr < NaNO_3 < NaI < NaSCN < NaCN$; (8) it is increased by those drugs which increase the excitability to potassium, viz. adrenaline, veratrine, trimethylamine; as a result of treatment with these drugs, the muscle may be rendered inexcitable to A.C., but contract strongly on cessation of the current. Cessation of the current which has been passed for 10 sec. may thus produce a tension of 20–40 g., though there is no visible response during the passage of the current. Such a phenomenon is reliably produced in potassium sensitive muscles if the chloride of the *Mytilus* saline is replaced with iodide, or if the muscle is treated with veratrine hydrochloride (1 in 800 in *Mytilus* saline, *p*H 7). This shows that profound changes may take place in a muscle, which apparently appears inexcitable to a given stimulus.

As with the potassium contraction, hypotonic *Mytilus* saline or sea water at first decreases and then increases the off-contracture, but the preliminary decrease may sometimes be absent; *Mytilus* saline rendered hypertonic by addition of sodium chloride has the opposite effect. If the osmotic pressure of sea water or *Mytilus* saline is increased by addition of glucose, then the off-contracture only diminishes. If the sodium chloride content of the surrounding medium is reduced by replacement with glucose, the off-contracture diminishes, though the tension produced by A.C. may increase.

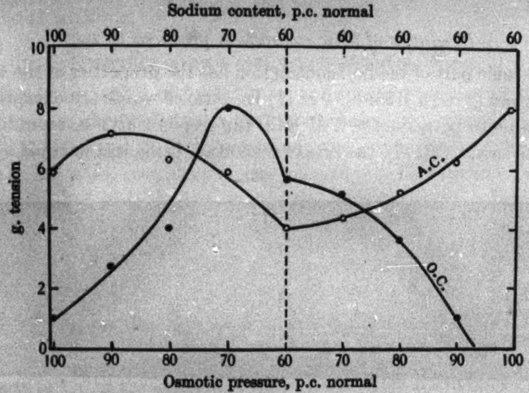

Fig. 1. Effect of osmotic pressure on the response to A.C. and the off-contracture (O.C.). The muscle was stimulated with A.C. (10 V.-5 min.). The tension produced by A.C. declines after a slight preliminary increase, the off-contracture increases. The osmotic pressure was then restored to normal by adding glucose; the tension produced by A.C. increased, while the off-contracture was abolished.

When the muscle is immersed in hypotonic *Mytilus* saline, it is subjected to two processes, first, decrease in the osmotic pressure, and secondly, diminution in the sodium chloride content of the medium; the opposite happens when the muscle is immersed in *Mytilus* saline rendered hypertonic by addition of sodium chloride. It appears that decrease in osmotic pressure augments the off-contracture, but decrease in the sodium chloride content diminishes it; similarly increase in osmotic pressure diminishes the off-contracture, but increase in the sodium chloride content of the medium augments it. When the muscle is immersed in a hypotonic or hypertonic solution, the effect on the off-contracture is determined by these two opposing factors. This is well seen from the experiment of Fig. 1. If the muscle is placed in *Mytilus* saline, rendered hypotonic by withdrawal of sodium chloride, the off-contracture increases, while the A.C. contraction decreases after a slight preliminary increase; if now the osmotic pressure is restored to normal by addition of glucose, the A.C. contraction is increased, while the off-contracture is abolished, though the sodium chloride content of the medium remains the same.

Properties of tonic contraction produced by D.C.

The tonic part of the D.C. contraction has the properties of the sodium contraction [Singh, 1938*b*], for: (1) In muscles which are exceptionally inexcitable to potassium (0·2 *M* KCl) the response to D.C. resembles the response to A.C. (Fig. 2), the rate of relaxation being just as rapid. If now the muscle is treated with any of the substances that increase the excitability to potassium, the rate of relaxation is decreased, and the typical D.C. response obtained. The rate of relaxation is also decreased, if such muscles are stimulated in calcium-free saline; or if this is not effective in both calcium and potassium-free saline or in 0·564 *M* NaCl. This in-

Fig. 2. D.C. contractions. The 1st two contractions are produced by A.C. 10 V.-10 sec. in muscle inexcitable to 0·2 *M* KCl, the 2nd two contractions are produced by D.C. 14 V.-10 sec. in muscles freshly arrived. The 5th contraction is produced by D.C. in another muscle from same batch of animals stored for 2 days; relaxation is not so rapid as in the 3rd and 4th contractions, but still unusually rapid for a D.C. contraction. The 6th contraction shows the effect of increasing the sensitivity of the muscle to potassium by replacing the chloride of the *Mytilus* saline with bromide. The 7th contraction is that of a muscle from another batch of animals and is produced in sea water; the arrow shows the point where the contraction ends and that produced by cessation of the current begins. The 8th contraction shows the effect of doubling the calcium and potassium content of sea water (1·8 c.c. of 0·56 *M* KCl + 2·8 c.c. of 0·376 *M* $CaCl_2$, sea water up to 100 c.c.) on the D.C. contraction; the tension increases but there is no contraction on cessation of the current.

variably converts the A.C.-like response into the typical response when the muscle is stimulated with D.C. (2) Conversely, in potassium sensitive muscles the response to D.C. is made to resemble the response to A.C. if the calcium and potassium content of *Mytilus* saline or sea water is doubled (0·02 *M* $CaCl_2$ and KCl); this is done by adding 1·8 c.c. of 0·564 *M* KCl and 2·8 c.c. of 0·376 *M* $CaCl_2$, to 95–100 c.c. of sea water. These concentrations of calcium and potassium together have the maximum inhibiting effect on the response to sodium chloride. By such treatment, the tension produced is also increased, when the muscle is stimulated with D.C.

Properties of tonic contraction produced by A.C.

Stimulation with A.C. (10 V.-10 sec.) ordinarily produces a twitch, but under certain circumstances a tonic contraction is produced. When the muscle is highly sensitive to potassium, the response to A.C. in *Mytilus* saline or sea water resembles the response to D.C. This corresponds to the fact mentioned above, that in muscles insensitive to potassium the response to D.C. resembles the response to A.C. Any significant deviation in the normal ionic composition of the *Mytilus* saline (0·01–0·02 *M* KCl and $CaCl_2$) may prolong the relaxation of the response to A.C. or any substance that produces a contracture may convert the A.C. twitch into a contracture.

In potassium sensitive muscles the response to A.C. may resemble the response to D.C. if the calcium of the *Mytilus* saline is excluded. If this is not effective exclusion of both calcium and potassium invariably converts the response to A.C. into a contracture. The same happens in 0·564 *M* NaCl, introduction of calcium (0·01–0·02 *M* $CaCl_2$) or calcium and potassium (0·01–0·02 *M* KCl) abolishes the contracture. It may be recalled that this concentration of calcium and potassium together have the maximum inhibiting effect on the sodium contracture. In pure NaCl solution, frog sartorius also responds to electrical stimulation by a contracture [Mines, 1908].

In potassium insensitive muscles, exclusion of calcium, potassium or immersion in 0·564 *M* NaCl does not convert the A.C. response into a "D.C. response", though the rate of relaxation may slightly decrease. Introduction of 0·02 *M* $MgCl_2$ or increase of *p*H to 7·8, *M*/300 borate buffer, may further prolong the relaxation. This concentration of magnesium and *p*H increase the excitability to potassium [Singh, 1938 *b*].

If excess of sodium chloride is added to *Mytilus* saline, the response to A.C. resembles the response to D.C. [Singh, 1938 *b*]. Relaxation is slow (*a*) in muscles from animals long stored in the refrigerator, (*b*) in muscles from cooled animals, (*c*) in muscles that have been immersed in *Mytilus* saline for a long time, especially if the *p*H of the latter is 7·8 (*M*/300 borate). It is significant to note that it is these types of muscle that contain excess of sodium [Singh, 1938*a*]. The effect of the above three procedures is less marked in potassium insensitive muscles. As a result of frequent stimulation with A.C. (10 V.-10 sec.), for about ½–1 hr., relaxation is greatly prolonged. Relaxation of frog muscle is also prolonged as a result of fatigue. The sodium content of frog muscle increases as a result of prolonged activity [Fenn, 1937].

Excess of ammonium (0·1–0·3 *M*) or potassium (0·03–0·1 *M*) converts the A.C. twitch into a contracture provided the muscle is not rendered inexcitable to A.C. in excess of potassium. The efficacy of these cations varies in the order $Na < NH_4 < K$, that is, the same as their stimulating power. Thus in six summer muscles, inexcitable to 0·1 *M* KCl, the concentration of ammonium chloride required to prolong the period of relaxation after a contraction giving 30–40 g. tension, from about 15–30 sec. in *Mytilus* saline to about 10 min., was 0·2–0·25 *M*, while the concentration of potassium chloride required was 0·03–0·035 *M*. In 0·05–0·07 *M* KCl, the period of relaxation was 60–90 min. To prevent inexcitability in excess of potassium, the concentration of calcium in the experimental solutions may be increased from 0·01 *M* to 0·02 *M* $CaCl_2$ (5·6 c.c. of 0·376 *M* $CaCl_2$ in 100 c.c. of *Mytilus* saline).

Fig. 3. Effect of barium on the response to A.C. (10 V.-10 sec.). The 1st three contractions are in *Mytilus* saline, the 4th and the 5th are in Ca-free saline, the 6th is in 0·014 *M* $BaCl_2$ in Ca-free saline and the 7th in 0·029 *M* $BaCl_2$ in same.

Excess of calcium or barium invariably converts an A.C. response into a "D.C. response" (Fig. 3), just as it invariably makes all muscles contract. Excess of calcium is less potent than excess of potassium in slowing the relaxation, just as it is less potent in stimulating the muscle. In sensitive muscles 0·03 M $CaCl_2$ may be sufficient to decrease the rate of relaxation, but in potassium insensitive muscles, the rate of relaxation may not be appreciably decreased in concentrations as high as 0·1–0·2 M $CaCl_2$. Like barium, excess of sodium cyanide (0·05 M) invariably converts an A.C. twitch into a contracture.

The effect of barium and sodium cyanide is less marked in potassium insensitive muscles; further, the effect of barium is more marked in the absence of calcium. Exclusion of calcium increases the sensitivity of the muscle to barium. In a muscle immersed in *Mytilus* saline (unbuffered, as the phosphate precipitates the barium) a tension of 25 g. produced by A.C. (10 V.–10 sec.) took about 15 sec. to subside; in the presence of 0·028 M $BaCl_2$ the muscle took 7 min. to relax by 50 %; when calcium was excluded it took 23 min. In the absence of calcium, besides that of A.C. and D.C., relaxation of most contractions is slow.

The anions bromide, nitrate, iodide, and thiocyanate also decrease the rate of relaxation, being more potent than chloride. Their effect in decreasing the rate of relaxation, just as their stimulating power, is much less than that of cyanide. The muscle, however, may be rendered inexcitable to A.C. in these anions, and in potassium insensitive muscles they are not very effective.

Excess of magnesium does not convert the response to A.C. into a contracture; it has also no stimulating powers.

Adrenaline (1 in 10^5), veratrine hydrochloride (1 in 3500), caffeine (1 in 100) also decrease the rate of relaxation (Fig. 4). Oliver & Schafer [1895] found the twitch of voluntary muscle in the frog and the dog was considerably prolonged after injection of suprarenal extract, an effect similar to that of veratrine. In *Mytilus* muscle, insensitive to potassium (0·15–0·2 M KCl), these drugs have no appreciable effect; adrenaline may in fact increase the rate of relaxation of an A.C. contraction. It may be recalled that such concentrations of these drugs have opposite effects on the excitability to A.C. in potassium sensitive and potassium insensitive muscles respectively.

It has been stated above that the rate of relaxation after a response to A.C. may be small in potassium sensitive muscles; the above-mentioned drugs and anions that decrease the rate of relaxation after an A.C. contraction increase the sensitivity of the muscle to potassium. Decrease in the rate of relaxation accompanying increased sensitivity of the muscle to potassium is shown by the effect of chloral hydrate (Fig. 4); chloral hydrate (1 %) does not produce any contraction but increases the sensitivity of the muscle to potassium and to stretch. Relaxation after a contraction produced by stretch in summer, potassium insensitive, muscles immersed in adrenaline (1 in 50,000), veratrine or caffeine (saturated solution in *Mytilus* saline) is also rapid: relaxation is more rapid in acid solution (pH 6·5–7) than in alkaline solutions (pH 7·8); excitability to potassium at this pH is at its maximum.

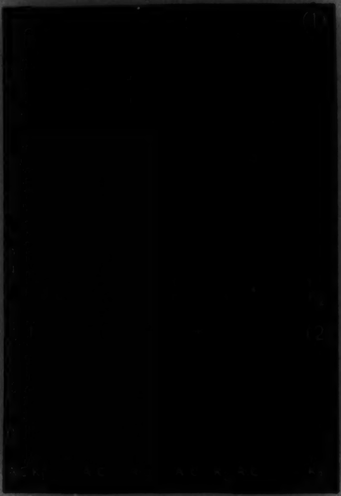

Fig. 4. Effect of veratrine and chloral hydrate on relaxation. (1) 1st two contractions by A.C. (10 V.-10 sec.) in *Mytilus* saline, 0·07 M KCl, only produced relaxation at K_1 after the 2nd contraction; effect of veratrine hydrochloride is shown by the 3rd contraction produced by A.C., and the 4th (drum not in motion) by potassium. Immersion for 15 min. (2) 1st contraction by A.C. (10 V.-10 sec.) in sea water; the muscle is inexcitable to potassium (0·07 M) at K_1; 2nd, 3rd and 4th contractions again by A.C. and the muscle is inexcitable to KCl at K_2; 5th contraction by A.C. in sea water containing 1 % chloral hydrate; the muscle is excitable to potassium at K_3. These muscles, though inexcitable to 0·07–0·01 M KCl, were, however, not exceptionally inexcitable to potassium, being stimulated by 0·1–0·12 M KCl.

The decrease in the rate of relaxation in excess of the ions referred to is probably due to increased sensitivity of the muscle to these ions during stimulation with A.C. [Singh, 1938b], as shown by the following facts: (1) the efficacy of these ions in prolonging the relaxation varies as their stimulating power, (2) if the muscle is not sensitive to chemical stimulation, then these ions are not so effective. One of the differences between contraction produced by A.C. in *Mytilus* saline and that produced by excess of the above ions is that the contraction produced by the latter is maintained; in sodium chloride, barium chloride and sodium cyanide the contraction is maintained till the death of the muscle [Singh, 1938*d*]. These ions then have two effects: (1) they produce contraction, (2) they maintain the contraction, not only if it is produced by themselves, but also if it is produced by any other agency, such as by an electrical stimulus.

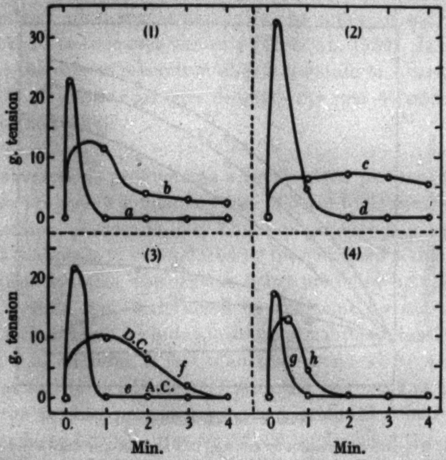

Fig. 5. Adaptation to A.C. and D.C. (1) Curve *a* by A.C. 10 V.-4 min. from potassium insensitive muscle from fresh animals, curve *b* in muscle from same batch of animals after 10 days' storage. (2) Curve *c* by A.C. in a muscle from an animal stored for 3 weeks, curve *d* from the same muscle after treatment with *Mytilus* saline, the NaCl content of which was reduced to 70 % of normal by replacement with glucose. Immersion for 30 min. (3) Curve *e* by A.C. (14 V.-4 min.) from a potassium insensitive muscle, curve *f* by D.C. (14 V.-4 min.). (4) Curve *g* by A.C. (10 V.-4 min.) in a muscle immersed in *Mytilus* saline for 10 min., curve *h* after immersion for 2½ hr. (*p*H 7·8-*M*/300 borate).

Prolongation of relaxation after the muscle has been stimulated by D.C. in *Mytilus* saline or sea water is probably due to increased sensitivity of the muscle to sodium chloride during stimulation, since the properties of the tonic part of the D.C. contraction seem to be similar to those of the sodium contraction. For: (1) The tension produced by a given D.C. stimulus is less than the tension produced by an A.C. stimulus of the same magnitude; it may be recalled that in excess of sodium, potassium, ammonium and calcium, the tension produced by A.C. stimulation diminishes. (2) In muscles which are exceptionally insensitive to potassium, the response to D.C. resembles that to A.C.; in such muscles the ions just mentioned have less effect. (3) Sodium chloride diminishes adaptation of the muscle to A.C.; adaptation to A.C. is also diminished under those conditions which bring about an increase in the sodium content of the muscle, viz. prolonged storage or cooling of the animals, or prolonged immersion in *Mytilus* saline. If the muscle is stimulated with D.C. for 5 min., sea water being rapidly run through the muscle chamber to avoid accumulation of gas bubbles, adaptation to D.C. is found to be very slow (Fig. 5). (4) Muscles tend to recover from the preliminary effects of excess cations; if a muscle is exposed to the action of excess sodium chloride, the excitability to A.C. is depressed, but the muscle tends to recover, and the excitability once more increases. Similarly, if the decreased rate of relaxation after a response to A.C. is due to the action of sodium chloride, then it would be expected that the muscle would recover from this action, and that the

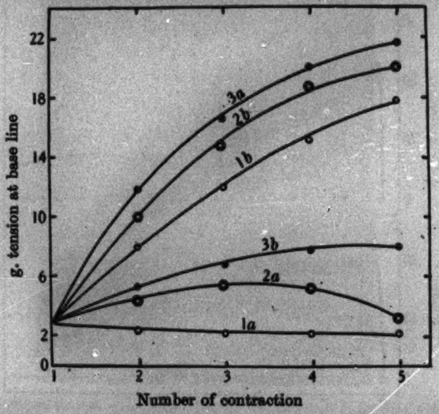

Fig. 6. Effect of frequent stimulation by A.C. and D.C. on base-line drawn through beginning of each contraction, stimulated by A.C. (10 V.-10 sec.) or D.C. (14 V.-10 sec.) every minute. Curve 1*a* by A.C. in 0·54 *M* NaCl + 0·01 *M* $CaCl_2$ + 0·01 *M* KCl, curve 1*b* in 0·56 *M* NaCl. Curve 2*a* by D.C. in 0·54 *M* NaCl + 0·01 *M* $CaCl_2$ + 0·01 *M* KCl; relaxation exceptionally rapid, the 4th contraction actually dropped the base-line, just as the usual effect of A.C. on a D.C. contraction. Curve 2*b* by D.C. 0·56 *M* NaCl. Curve 3*a* by D.C. in sea water, curve 3*b* by D.C. in sea water, the calcium and potassium content of which had been doubled (1·8 c.c. of 0·564 *M* KCl + 2·8 c.c. of 0·376 *M* $CaCl_2$, sea water up to 100 c.c.).

rate of relaxation would increase once more if the muscle is stimulated repeatedly at short intervals of time; this is actually observed. (5) Recovery from the effect of excess sodium chloride is promoted by calcium and is small in potassium sensitive muscles. Similarly, it is found that the drop in the base-line during repeated stimulation with A.C. is promoted by calcium. In 0·564 *M* NaCl the base-line may not drop at all; in 0·55 *M* NaCl+0·01 *M* $CaCl_2$ the base-line drops as usual (Fig. 6). (6) In potassium insensitive muscles adrenaline (1 in 10^5) antagonizes this rise in base-line; adrenaline antagonizes the action of sodium [Singh, 1938*b*] and reduces tone and viscosity [Winton, 1930]. It is significant to note that the rate of relaxation after a response to A.C. stimulation is decreased by all substances that decrease the rate of extension of the muscle [Singh, 1938*c*].

DISCUSSION

Tonic contractions produced as a result of the passage of an electric current have properties similar to those of other tonic contractions, produced by sodium, potassium, etc. The response to D.C. is extremely interesting, as it appears to be produced by the combined action of the tonic and the phasic systems, the tension being produced by the phasic, or potassium, system, but maintained by the tonic, or sodium, system. Since these two systems are antagonistic, the maximum tension produced by a given D.C. stimulus is less than that produced by a similar A.C. stimulus (instead of being greater, were the two systems synergistic). Moreover, the tension produced by a D.C. stimulus is increased if the muscle is rendered less sensitive to sodium by doubling the calcium and potassium content of sea water or *Mytilus* saline.

During stimulation with A.C. or D.C. the muscle is exposed to the action of sodium or chloride, but the resulting effect is more intense with D.C. than with A.C. These differences between the response to A.C. and D.C. may be explained by a scheme similar to that put forward by Winton [1937]. With each forward pulse of the A.C. we presume that the sodium or chloride ions move from the solution into the muscle membrane and with the reverse pulse the ions move back. In the case of unidirectional currents the ions move into the muscle membrane with the current, but have to diffuse back. This view is supported by the action of calcium, which is known to decrease the permeability of membranes. Decrease in the calcium content or increase in the sodium chloride content of *Mytilus* saline decreases the rate of relaxation.

The effect appears to be produced by sodium and not chloride, as the muscle recovers from the effect of excess cations, but not from that of the anions. From this view it would be expected that the sodium content of the muscle should increase as a result of stimulation. But if the effects are produced by a few ions only, such as with acetylcholine [Clark, 1933], then no appreciable increase in the sodium content might be produced; if, however, stimulation be continued long enough, then an appreciable increase in the sodium content of the muscle might take place.

Many workers have found that the sodium content of muscle increases as a result of activity (for discussion see Fenn, 1936). The sodium or the total base content of *Mytilus* muscle increases if it is immersed for a long time in *Mytilus* saline, if the animals are cooled or stored for long in the refrigerator, or if the muscle is immersed in calcium-free saline; under these conditions relaxation becomes prolonged. The sodium content of unstriated muscle is greater than that of striated muscle, and the former is more viscous and relaxes more slowly than the latter.

SUMMARY

1. Properties of the alternating current off-contracture are those of the potassium contraction.

2. Properties of the tonic part of the direct current contraction are those of the sodium contraction.

3. Substances that produce contracture prolong the relaxation of an alternating current contraction in potassium sensitive muscles.

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FACTORS IN SEXUAL-SKIN OEDEMA

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According to Collings [1926] cyclical reddening of the sexual skin in the rhesus macaque (*Macaca mulatta*) as a result of oestrogenic stimulation is due to engorgement of a plexus of large thin-walled blood channels that lies just beneath the epidermis, and he ascribes such swelling as occurs in the sexual skin of this species to an unspecified change in the subepithelial tissues, and not to oedema. On the other hand, Zuckerman & Parkes [1932] failed to find definite differences between the blood vessels of the swollen and inactive sexual skin of the Hamadryas baboon (*Papio hamadryas*), whereas changes in the cell density of the subcutaneous tissues (determined by estimates of the number of nuclei per cubic millimetre of tissue) suggested that the swelling does result from oedema. The material used in this latter study precluded detailed histological investigation, and "on general grounds" the dispersion of the nuclei was ascribed to an accumulation of intercellular fluid rather than to cellular enlargement.

That the process of sexual-skin swelling is, in fact, a physiologically regulated oedema is proved by observations that were made on the pig-tailed monkey (*Macaca nemestrina*, Krohn & Zuckerman [1937]). Body weight in the female of this species fluctuates with the phases of the sexual skin, and when the skin is fully swollen, body weight may be 17 % greater than it is when the skin is inactive. Investigation of daily changes in water metabolism showed that the increase in weight can be largely accounted for by the accumulation and retention of fluid in the sexual-skin area. The conclusion that the water is mainly held in the sexual-skin area is supported by the observation [Krohn & Zuckerman, 1937] that the swollen sexual skin of baboons, when removed at autopsy, may weigh from 10 to 28 % of the total body weight. Most of the retained fluid appears to be extracellular, for when the swollen tissue is incised, clear and moderately thick fluid ("sexual-skin exudate") drips freely from the exposed surfaces. Moreover, when swollen sexual skin is removed at autopsy, frozen, and then sectioned with a knife, the cut surfaces are found to be largely made up of crystals of ice. Observations on the water content of "drained" sexual-skin tissue and on the osmotic pressure of both blood serum and sexual-skin exudate suggest, however, that water is also taken up by the connective tissue elements of the sexual skin [Fisher & Zuckerman, 1937]. This finding implies that the cells increase in size during the process of sexual-skin swelling, and the following study was carried out primarily in order to discover whether or not such a cytological change does in fact occur.

MATERIAL AND METHODS

The sexual skin in the female rhesus monkey (*M. mulatta*) undergoes a process of maturation in which there normally occur three well-differentiated phases [Zuckerman *et al.* 1938]. During the first phase (puberty) the conspicuous change is swelling, and oedema is limited to the circumgenital area. During the second phase (adolescence) the oedema gradually disappears from the central circumgenital area, which takes on an indurated appearance, and the zone of oedema extends peripherally. During the third phase (maturity) swelling does not occur, and the prominent change is reddening, the main zone of coloration being restricted to the genital region. The process of sexual-skin maturation is dependent not on age but on the extent to which the sexual skin is subjected to the influence of oestrogenic hormone.

The genital and circumgenital skin of the immature male rhesus monkey does not normally show any changes, but it can be transformed in the same way as in the female by means of oestrin injections.

The tissues used in the present study were taken at autopsy from both normal and gonadectomized immature or adolescent male and female rhesus monkeys (*M. mulatta*). The animals had been injected for different periods of time with oestrone, and the sexual skins that were studied were representative of the three types of reaction observed during the normal process of maturation. Inactive control tissues were also studied. Details of the animals are given in Table I.

Specimens were taken from different regions of the genital and circumgenital areas (see Tables II and III). A piece of each specimen was fixed in Bouin's fluid and was subsequently stained with haematoxylin and eosin. One or more of the following staining methods were also used on

Table I. Details of animals used in present study. Autopsies were in all cases performed on the day following the last injection

Animal no.	Sex	Maximum body wt. g.	Experimental treatment	State of sexual skin
55	Female	3970	Spayed, no oestrone injections	Completely inactive
96	,,	3200	Spayed, up to 300 μg. oestrone daily for 14 days	Pubertal "blister" swelling
218	,,	2260	100 μg. oestrone daily for 48 days	Adolescent type of response
98	,,	3800	Spayed, 100 μg. oestrone daily for 365 days	Mature type of response
376	Male	2540	Nil	Immature control
124	,,	2840	100 μg. oestrone daily for 14 days	Pubertal "blister" swelling
196	,,	4040	100 μg. oestrone daily for 15 days	,,
257	,,	2200	100 μg. oestrone daily for 14 days	,,
258	,,	—	,,	,,
101	,,	3130	100 μg. oestrone daily for 62 days	Adolescent type of response
197	,,	3690	100 μg. oestrone daily for 55 days	,,
115	,,	2940	100 μg. oestrone daily for 368 days	Mature type of response

some of these specimens: iron alum haematoxylin, Mann's methyl blue eosin, Mallory's connective tissue stain, Weigert's elastin stain, Van Gieson and acid fuchsin; certain specimens were fixed in Zenker-formol, and stained for mucin with mucicarmine, toluidin blue, and mucisudan.

Cytological preparations were also made by the Aoyama, Champy and Weigl methods.

Observations

(*a*) *General*

Before it has ever responded to oestrogenic stimulation, the sexual skin of the immature rhesus monkey differs little in histological appearance from skin that lies outside the genital zone. The rete mucosum is somewhat wider and less crenated than usual, and the dermal vessels appear to be more numerous and in general more dilated than in ordinary skin (see below). The dermal connective tissue cells are small and crowded together, and they have relatively little cytoplasm. They are mainly fibroblasts, but mast cells can also be recognized. Mitoses are rarely seen in either the dermis or epidermis—even in specimens in which dividing cells have been "fixed" by means of the injection of colchicine previous to death.

During its first (pubertal) phase of activity, the sexual skin undergoes more pronounced changes than it does in later reproductive life. The number of layers of cells in the rete mucosum may increase to as many as six, and numerous mitoses are to be seen in its deeper layers. The cells themselves do not appear to be larger than normal. The superficial layers of the stratum corneum are in process of desquamation, and, as **Collings** noted, the boundary between dermis and epidermis is straighter than in inactive skin.

During the pubertal phase of sexual-skin swelling the dermis becomes much thicker than normal, and its cellular elements become widely dispersed and separated by numerous intercellular spaces (many of which appear to be spherical). Occasional mitoses are to be seen in the connective tissue cells, which are also individually much larger than normal (see below). The large size of the fibroblasts in active sexual skin was noted by **Bachman** *et al.* [1935]. Fibroblasts with two nuclei are also sometimes observed. The white fibres appear to be split up and oedematous, and the abundant fibres of elastic tissue appear to be thinner than in inactive skin.

During adolescence and maturity the epidermis of the active sexual skin is in general the same in appearance as in puberty. The histology of the dermis alters, however, as the skin matures. During adolescence, when the "pubic lobes" cease to swell like a blister [**Zuckerman** *et al.* 1938], the connective tissue cells are less widely dispersed and less swollen than they are during puberty. When the sexual skin is fully mature no very obvious changes appear to occur except in the vessels.

(*b*) *Size of connective tissue cells*

The changes that take place in the connective tissue cells of the dermis are demonstrated in Pl. I, figs. 1 and 2. Tables II and III give the mean cross-sectional areas of connective tissue cells in different regions of the sexual skin. The terms by which these regions are designated are those used by **Zuckerman** *et al.* [1938]. Ten cells were taken at random in each piece of skin and drawn with the help of a camera lucida at a magnification of 1680 diameters. Areas were measured with a planimeter. The mean dimensions of normal control cells have been taken as unit size. No special attempt was made to differentiate the connective tissue cells measured; most, if not all, were fibroblasts.

It will be seen that during the pubertal phase of sexual-skin activity the cross-sectional area of the connective tissue cells of the circumgenital skin in both the male and female rhesus may become four to six times larger than normal. The cells also increase in size, but not as much, during the adolescent phase of sexual-skin activity, when the circumgenital skin itself swells only slightly. They do not appear to enlarge at all when the reaction of the sexual skin to oestrogenic stimulation has become mature. The cells enlarge not only throughout the genital and circumgenital areas, but also in the connective tissue of the nipple, which responds to oestro-

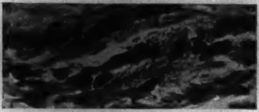

Fig. 1.

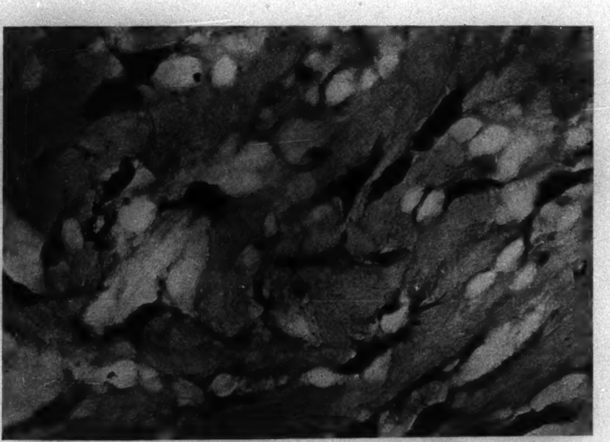

Fig. 2.

Table II (Female). Mean cross-sectional area of connective tissue cells in different parts of sexual skin. Cell areas are expressed as multiples of the corresponding areas in control specimens. The figures given in brackets are the actual mean areas in cm.² of the camera lucida tracings which were drawn at a linear magnification of 1680. For further details see text

	Animal no.		Margin of sexual skin	Scrotal lobes	Central ridges	Skin anterior to callosity	Skin posterior to anus	Base of tail	Nipple
Control	55	Cell body	1·0 (0·46 ±0·048)	1·0 (0·48 ±0·039)	1·0 (0·66 ±0·072)	1·0 (0·47 ±0·050)	1·0 (0·65 ±0·048)	1·0 (0·68 ±0·102)	1·0 (0·51 ±0·050)
		Nucleus	1·0 (0·28 ±0·002)	1·0 (0·37 ±0·033)	1·0 (0·48 ±0·056)	1·0 (0·36 ±0·045)	1·0 (0·50 ±0·036)	1·0 (0·44 ±0·126)	1·0 (0·39 ±0·037)
Pubertal swelling response	96	Cell body	5·4 (2·49 ±0·259)	5·8 (2·78 ±0·214)	6·1 (4·01 ±0·367)	6·6 (3·10 ±0·370)	4·3 (2·82 ±0·289)	4·2 (2·84 ±0·039)	4·8 (2·45 ±0·159)
		Nucleus	2·6 (0·74 ±0·082)	2·6 (0·95 ±0·040)	2·2 (1·05 ±0·066)	3·4 (1·21 ±0·109)	2·5 (1·25 ±0·088)	2·4 (1·04 ±0·072)	2·3 (0·91 ±0·088)
Adolescent response	218	Cell body	—	2·2 (1·05 ±0·044)	3·2 (2·08 ±0·254)	—	—	3·3 (2·21 ±0·184)	1·0 (0·55 ±0·060)
		Nucleus	—	1·6 (0·61 ±0·130)	1·8 (0·84 ±0·124)	—	—	1·9 (0·83 ±0·042)	0·8 (0·32 ±0·041)
Mature response	98	Cell body	2·0 (0·94 ±0·108)	0·9 (0·42 ±0·045)	0·8 (0·52 ±0·056)	1·4 (0·65 ±0·054)	0·9 (0·62 ±0·053)	0·7 (0·49 ±0·046)	1·1 (0·58 ±0·051)
		Nucleus	2·0 (0·57 ±0·062)	0·8 (0·28 ±0·033)	0·6 (0·28 ±0·028)	1·2 (0·42 ±0·053)	0·8 (0·40 ±0·040)	0·7 (0·33 ±0·036)	1·0 (0·39 ±0·044)

Table III (Male). Mean cross-sectional area of connective tissue cells in different parts of sexual skin. Cell areas are expressed as multiples of the corresponding areas in control specimens. The figures given in brackets are the actual mean areas in $cm.^2$ of the camera lucida tracings which were drawn at a linear magnification of 1680. For further details see text.

	Animal no.		Prepuce	Dependent part of scrotum	Lateral margin of scrotum	Skin posterior to scrotum	Side of base of tail
Control	376	Cell body	1·0 (0·55 ±0·070)	1·0 (0·74 ±0·043)	1·0 (0·65 ±0·070)	1·0 (0·57 ±0·056)	1·0 (0·65 ±0·130)
		Nucleus	1·0 (0·35 ±0·043)	1·0 (0·40 ±0·049)	1·0 (0·37 ±0·040)	1·0 (0·35 ±0·042)	1·0 (0·37 ±0·056)
Pubertal swelling response	124	Cell body	—	3·8 (2·83 ±0·310)	4·1 (2·64 ±0·173)	3·6 (2·06 ±0·176)	5·4 (3·50 ±0·314)
		Nucleus	—	2·2 (0·88 ±0·074)	2·3 (0·86 ±0·048)	2·0 (0·70 ±0·047)	3·2 (1·20 ±0·099)
	196	Cell body	4·2 (2·33 ±0·261)	3·8 (2·79 ±0·269)	—	5·0 (2·85 ±0·237)	2·8 (1·83 ±0·247)
		Nucleus	2·3 (0·81 ±0·064)	2·3 (0·92 ±0·065)	—	2·6 (0·90 ±0·056)	1·7 (0·63 ±0·047)
	257	Cell body	—	2·5 (1·88 ±0·288)	—	—	—
		Nucleus	—	1·7 (0·69 ±0·079)	—	—	—
	258	Cell body	—	2·7 (2·03 ±0·244)	—	—	—
		Nucleus	—	2·0 (0·79 ±0·099)	—	—	—
Adolescent response	101	Cell body	2·3 (1·27 ±0·114)	2·6 (1·96 ±0·176)	3·4 (2·19 ±0·223)	—	3·3 (2·15 ±0·260)
		Nucleus	1·8 (0·63 ±0·052)	2·8 (1·11 ±0·091)	2·5 (0·94 ±0·083)	—	2·1 (0·79 ±0·100)
	197	Cell body	1·6 (0·86 ±0·081)	0·9 (0·64 ±0·003)	—	1·1 (0·63 ±0·103)	2·0 (1·32 ±0·209)
		Nucleus	1·5 (0·54 ±0·066)	1·0 (0·39 ±0·048)	—	1·0 (0·36 ±0·046)	1·6 (0·58 ±0·085)
Mature response	115	Cell body	0·7 (0·40 ±0·040)	0·6 (0·48 ±0·047)	0·8 (0·55 ±0·069)	—	—
		Nucleus	0·8 (0·29 ±0·088	0·7 (0·28 ±0·002)	0·9 (0·32 ±0·041)	—	—

genic stimulation in much the same way as the circumgenital sexual skin. The nuclei of the connective tissue cells also enlarge, although relatively less than do the cell bodies.

The available figures provide only a two-dimensional view of the process of cellular enlargement, and it is obvious that the actual enlargement must be greater than is disclosed by our measurements.

The fact that part of the water of the sexual skin is intracellular may help to explain why the swollen skin does not pit on pressure in the same way as does an oedematous region in cardiac failure. It may be noted too that enlargement of connective tissue cells does not apparently occur in ordinary oedema. For example, oedematous skin from several regions, including sexual skin, of a monkey (286) which became oedematous for reasons that were not discovered was examined, and it was found that the fibroblasts were no larger than is usual either in normal skin or in inactive sexual skin.

(c) *Blood vessels.*

Collings was led by his observations on the rhesus monkey to the view that there is a direct correlation between the degree of reddening of the sexual skin and the size and engorgement of the superficial blood vessels. According to him the deeper vessels of the dermis of the sexual skin are larger than is usual in unspecialized skin, and contain more blood when the skin is active. The papillary layer has a very rich vascular supply, and its arterioles have a wider lumen than ordinary arterioles. As these arterioles reach the base of the epidermis, "the walls become thinner like those of capillaries, while the lumina become relatively larger and in many cases actually larger....In fact, these thin-walled blood channels close beneath the epithelium are frequently larger than the feeding arterioles deeper beneath in the subcutaneous tissues."

We have confirmed these observations by means of projector tracings of sections of many different regions of active and inactive sexual skin, and have also observed that the number of vessels seen in inactive sexual skin is much greater than is found in non-sexual skin. These histological observations have also been amplified by the following experiments:

(1) An immature rhesus monkey (385), whose sexual skin had been made oedematous by a series of oestrone injections, was perfused with saline under deep nembutal anaesthesia. When the returning saline appeared to be free of blood, India ink (diluted with four times its volume of saline) was injected into the descending aorta. The skin of the genital region showed the effects of the injection before any other part of the

body surface, and at the end of the experiment the region immediately ventral to the vulval cleft, the swollen vulval lips, the perinaeum proper, the everted anus, the swollen circumanal region and the base of the tail were blacker than any other part of the body surface. They were also marked off clearly from adjacent skin. The swollen pubic lobes and central ridges did not take the "dye". When the skin of the genital region was incised it was found that the subcutaneous tissues of the regions which were superficially black were stained and had blood vessels which were marked by the injection.

(2) Chicago blue was used in a second injection. Estimations of the rate of its disappearance from the blood plasma of dogs [Dawson *et al.* 1920] or of its diffusability through water and gelatin [Rous *et al.* 1930] show that this substance is one of the more useful dyes available for studying the relative density of the vascular bed in different areas of skin. A mature female rhesus (393) with an inactive sexual skin was used in this second experiment. After perfusion with warm saline, 50 c.c. of a 1% solution of the dye were injected into the descending aorta. The first part of the body surface to show the dye was the circumanal region and the ventral surface of the base of the tail. At the end of the injection the whole sexual-skin area was clearly marked off by its deeper blue colour from the surrounding skin. The iliac region was also intensely coloured, and graded off into paler blue above the level of the iliac crests.

The skin was dissected from the underlying muscle and selected pieces of sexual and non-sexual skin were cleared and prepared as "mass" specimens. The greater vascularity of the sexual skin was clearly demonstrated by this method.

(3) Another animal (215) was slowly perfused when under deep anaesthesia before autopsy with saline at 46° C., and then injected through the abdominal aorta first at a pressure of 100 mm. and then at 150 mm. Hg with gelatin-carmine (the injection being made in a specially warmed room). The carcase was completely bloodless before the gelatin-carmine was introduced, and the sexual skin was no different in colour from its surroundings. The circumanal region reddened first, and the whole sexual-skin region then became a vivid red, which was well demarcated from the adjacent pink skin. The specimen was placed in 90% alcohol in a refrigerator, and frozen and paraffin sections of selected regions were prepared after 2 days. A very clear picture was obtained of the differences between the vascular supply of sexual and non-sexual skin and an equally clear demonstration of the very large subepidermal capillaries (or sinusoids) noted by Collings. The blood can be entirely and permanently

moved from these vessels in a fresh carcase by firm stroking with the handle of a knife.

(4), (5) Two other animals, after saline perfusion at autopsy, were injected with methylene blue. The whole sexual skin immediately became blue and demarcated from its surroundings. The change was much more rapid than in the experiments recorded above. Methylene blue diffuses very rapidly, and it is possible that this observation implies that the sexual-skin vessels are not only more numerous and dilated than those of normal skin, but also more permeable (see below).

The active and hyperaemic sexual skin is a warm and flushed skin, a fact which indicates both arteriolar and capillary dilatation. Collings's observations which, as noted above, we have confirmed, provide histological evidence in favour of this conclusion, which further suggests that capillary pressure, and therefore mean filtration pressure, may be increased during phases of oestrogenic activity. This is a factor which predisposes to the transudation of excess fluid into the intercellular spaces of the sexual skin. Whether or not oestrogenic substances have a direct dilator action on vessels or whether the dilatation is due to metabolites is still to be determined, and in this connexion it is significant that oestrogenic substances act as well on denervated as on normal sexual skin [Zuckerman, 1935; Bachman *et al.* 1936; Zuckerman *et al.* 1938].

Oedema of the sexual skin, while it occurs throughout life in such species as baboons, the chimpanzee and the pig-tailed macaque, occurs as a rule only during puberty and adolescence in the rhesus. Hyperaemia of the sexual skin continues throughout life in this species, and the non-occurrence of oedema in mature animals must, therefore, be related either to some change in the large sub-epidermal capillaries or sinusoids, to some change in the connective tissue elements, or to an increase in lymphatic drainage.

(*d*) *Mucoid reactions*

Erection of the cock's comb, which is dependent on male hormone stimulation, is due to the secretion of mucoprotein into the intercellular spaces [Hardesty, 1931]. It is, therefore, of interest to enquire whether or not a similar secretion of mucoprotein is in any way concerned in the process of sexual-skin swelling. The gelatinous substance which exudes from the intermediate layer of a cut cock's comb gives a mucoid reaction, and the turgidity of the cock's comb, as compared with that of the capon and hen, is due to the presence of larger quantities of intercellular mucoid, believed to be secreted by fibroblasts. Mucoid is not secreted in the peripheral zone of the comb, and it is suggested by Hardesty that the secretion in the intermediate layer is due to the direct effect of androgenic stimulation on the fibroblasts. Hardesty's detailed account of the histological basis for the responses of the comb to endocrine stimulation contains no reference to variations in the water metabolism of the comb.

In investigating the mucoid reactions of the inactive and active sexual skin we have had the co-operation of Mr E. H. Leach. The intercellular spaces in both active and inactive sexual skin of rhesus macaques give a negative reaction, whereas in the active skin the interspaces of the collagen fibres, like the fresh exudate of the skin, give a very faint positive response. The main difference between the two types of skin lies in the fact that mast cells filled with granules of what is generally believed to be mucoprotein are present in the inactive phase, and appear to be partly depleted of their granules when the skin is oedematous. The change is most marked in regions away from blood vessels. This observation, which is being investigated further, suggests that in phases of oestrogenic stimulation the mast cells discharge mucoprotein into the intercellular spaces. This mucoprotein may be able to hold some of the intercellular water.

The apparent discharge by the mast cells of granules during phases of oestrogenic stimulation may partly explain the observation that the protein concentration of a mixture of sexual-skin exudates from three immature rhesus monkeys which were in precisely the same condition of swelling proved to be as high as 2·58% [Fisher & Zuckerman, 1938]. The mean protein content of the corresponding blood sera was 6·34%. The mean protein content of oedema fluid rarely rises above 1% except in inflammatory conditions [Landis, 1934; Peters, 1935].

(e) *The passage of dye through the sexual skin*

It has recently been suggested [Clark & Clark, 1933; Bensley, 1934; MacMaster & Parsons, 1938] that under normal conditions interstitial substance is a gel and that it becomes fluid only when oedema occurs. Sexual-skin swelling clearly appears to represent a process of oedema, but in order to test the possibility that its interstitial substance is a gel capable of holding fluid by imbibition, we made the following observations, which are based on the view, stated by Peters [1935], that rapid passage of a dye through tissues would not be possible if the substance of the intercellular spaces were a gel. 1 c.c. of a dilute solution of trypan red was injected into each side of the swollen scrotum of an immature male rhesus monkey that had been injected daily for 15 days with

100μg. of oestrone. The injection was made superficially on one side, and about 1 cm. deep to the surface on the other. The animal was killed by means of deep nembutal anaesthesia 1 hr. and 40 min. later. It was found that the dye had diffused widely, and that the subcutaneous tissue of both sides of the scrotum was a homogeneous pink colour. The lymphatics leading to the inguinal glands on the side where the injection had been made deeply were more highly coloured than on the other; the glands themselves were not coloured on either side. The coloration of the subcutaneous tissues (and "sexual-skin exudate") did not extend posterior to the scrotum. Similar observations were made, using smaller quantities of a more concentrated solution of dye, and examining the subcutaneous tissues 50 min. after injection, on a prepubertal female which had received its first course of oestrone injections and whose sexual skin showed the characteristic "blister-swelling" that results from such stimulation [Zuckerman *et al.* 1938]. In this instance the spread, though obvious, was not as great as in the first experiment. The results of these experiments thus confirm the view, indicated by the observations of Clark & Clark and MacMaster & Parsons, that when it is swollen with oedema, the interstitial substance of the sexual skin is not a gel which prevents the passage of dye.

Control observations were made on five other rhesus monkeys, the first an immature normal male with a normal scrotum, the second and third immature females with inactive sexual skins, and the fourth and fifth mature females with typical unswollen sexual skins. In all cases it was found at autopsy that the dye had spread far less than in the two animals with active sexual skins, and that it was still mostly localized at the site of injection. Where observations were made between 40 and 60 min. from the time of injection, the spread was little greater than it was in a sixth animal that was injected after death.

DISCUSSION

The histological observations reported above are in keeping with the view, put forward previously on the basis of observations on the water content of drained and undrained swollen and inactive sexual skin, that sexual-skin oedema involves an increase in the water content of connective tissue cells [Fisher & Zuckerman, 1937]. The change in cell size reflects an alteration in cellular metabolism which occurs under the influence of oestrogenic stimulation. Oestrogenic substances can be demonstrated in active sexual skin [Fisher *et al.* 1936], and presumably they affect the cells directly.

The osmotic pressure of the tissue fluid of swollen sexual skin is relatively high. In three of eleven readings it proved to be greater than the osmotic pressure of the blood serum (7·1, 8·4 and 11·7 atm. as compared with 7·0, 7·4 and 7·9 atm. for the corresponding blood sera, **Fisher & Zuckerman** [1937]). The high pressure cannot be explained as being due to concentration resulting from an increase in cell water content, for if the reabsorption rate remains constant, the capillary transudation which would be stimulated by this change alone would restore the tissue fluid of the sexual skin to its normal proportions and osmotic pressure.

On the other hand, the high protein content of the interstitial fluid suggests that the capillary bed of the sexual skin becomes freely permeable under the influence of oestrogenic stimulation. The excess of tissue fluid in a swollen skin may, therefore, be due in greater part to increased permeability, and to the consequent diminished effective reabsorptive force of the colloid osmotic pressure of the blood plasma. The extent to which non-plasma proteins contribute to the high protein content of the interstitial fluid has, however, still to be determined, as have also the factors responsible for the fluid's high osmotic pressure.

A further problem which awaits investigation is the possibility that the stromal changes which occur in the endometrium during the menstrual cycle, and which are also related to oedema, are determined in the same way as the cellular changes of the sexual skin. Some observations on the intercellular substance of the stroma have already been published by **Bensley** [1934], who has shown, by means of toluidine blue, that a metachromatic ground substance (possibly allied to mucoprotein) is present only during the active proliferative phase and that it disappears when oedema develops in the stroma.

SUMMARY

1. Histological examination of different parts of the sexual skin of rhesus monkeys shows that when the skin is active and swollen the intercellular accumulation of fluid is associated with a considerable increase in the size of the connective tissue elements of the skin. On the basis of previous observations on the osmotic pressure of the interstitial fluid [**Fisher & Zuckerman**, 1937], the swelling of the cells may at least partly be ascribed to the taking up of water.

2. Perfusion experiments in which India ink, Chicago blue, carmine gelatine and methylene blue were used show that the vessels of the sexual-skin area are more dilated and numerous than the vessels of other parts of the body surface. Direct observations and histological study show that

hyperaemia of the sexual skin occurs during phases of oestrogenic stimulation.

3. Trypan red passes much more quickly through the subcutaneous tissue of the swollen sexual skin than through that of unswollen, inactive skin.

4. The mast cells apparently discharge granules during phases of swelling. This observation may be related to the fact that the protein concentration of the interstitial fluid of the swollen skin is relatively very high (2·58 % as compared with 6·34 % for corresponding blood serum).

Our warmest thanks are due to Mr E. H. Leach for his help in investigating the mucoid reactions of the sexual skin, and to Dr B. G. Maegraith for performing the carmine-gelatin injection (p. 20). We are also very grateful to Dr R. B. Fisher for his critical interest in the work, which was assisted by a grant to S. Z. from the Nuffield Medical Committee, Oxford. The cost of animals, most of which were primarily used in collateral investigations, was defrayed out of funds provided by the Medical Research Council.

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EXPLANATION OF PLATE I

Fig. 1. A section of the dermis of the "pubic lobes" of the inactive sexual skin of an adolescent female rhesus monkey (55). Between fifteen and twenty connective tissue cells are shown. ×445.

Fig. 2. A comparable section taken from an adolescent monkey (96) whose "pubic lobes" were swollen with oedema. Approximately the same number of cells are shown. ×445.

The photomicrograph demonstrates the dispersal of the cells by intercellular oedema and also the increase in the size of the cell bodies and nuclei. The difference in size between the two prints gives a two-dimensional picture of the amount of swelling that can occur in the "pubic lobes" of the sexual skin.

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THE EFFECT OF HIGH DOSES OF ANDROGENIC SUBSTANCES ON THE WEIGHTS OF THE TESTES' ACCESSORY REPRODUCTIVE ORGANS AND ENDOCRINE GLANDS OF YOUNG MALE GUINEA-PIGS

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Implantation studies have shown that prolonged administration of oestrogenic hormones will decrease the gonadotropic potency of the pituitary [e.g. Meyer *et al.* 1932]. This has been demonstrated in another way by Moore & Price [1930, 1932] and Zondek [1936*a*] who showed that testicular atrophy resulted from the administration of oestrogenic hormones to immature male rats. Similar results with mature males were obtained by Halpern & D'Armour [1936]. It was reasonable to suppose that the damage to the testes was due to suppression of the gonadotropic function of the hypophysis by the action of the oestrogens, since injections of the latter caused no testicular damage if pituitaries were implanted at the same time [Moore & Price, 1932]. In the mature female rat administration of oestrogens leads under some conditions to decrease in gonad size and weight [Selye *et al.* 1935], but often to the reverse [Selye *et al.* 1935; Bacsich & Folley, 1938] on account of the heavy luteinization, presumably due to increased secretion of the luteinizing hormone by the anterior pituitary [Fevold *et al.* 1936].

Androgenic hormones appear to exert an analogous suppressing action on the secretion of the hypophyseal gonadotropic hormone, since administration of testis extracts [Ihrke & D'Amour, 1931] and of crystalline testosterone and androsterone [Browman, 1937] inhibits the oestrous cycle in the female rat. Similarly Zuckerman [1937] has shown that injections of testosterone propionate will inhibit the menstrual cycle in the female monkey. In the young male, injections of androgenic extracts lead to testicular atrophy [see Moore & Price, 1930, 1932, for the effect of testicular extracts; Schoeller & Gehrke, 1933; Itho & Kon, 1935, for the effect of urinary androgens]. Moore & Price further showed that the simultaneous administration of anterior pituitary extracts prevented the testicular atrophy due to the action of the androgenic extracts, indicating that, as in the case of oestrogenic hormones, the androgens affected the testes through the pituitary. It is interesting to note that Korenchevsky *et al.* [1937] reported only relatively slight regression of the testes in adult male rats which had received high daily doses of testosterone and its propionate.

This paper describes the effects of the administration of various crystalline androgens on the weights of the testes, accessory reproductive organs and endocrine glands of immature male guinea-pigs. It is hoped to describe in another paper the histological changes, particularly in the pituitary, resulting from the injections.

EXPERIMENTAL

The experiments were carried out on immature male guinea-pigs of mixed strain averaging 170–190 g. body weight at the beginning of the experiment. For most treatments groups of five were used, though in three instances one animal died. Thus the group receiving injections of 17-methyltestosterone, which, owing to scarcity of material originally consisted of four animals, became reduced to three during the experiment. All members of each experimental group received daily injections of 2 mg. androgen dissolved where possible in 0·2 ml. sesame oil. In experiments involving testosterone propionate the daily dose was 2·36 mg. (≡2·0 mg. testosterone). In the case of the "diols", 0·8 ml. oil was necessary to hold the daily dose in solution. In order to study the effect of oil volume, experiments with testosterone and testosterone propionate were carried out at both of the above-mentioned dilutions. Two control groups received daily injections of 0·2 and 0·8 ml. sesame oil respectively.

The guinea-pigs were kept under as uniform conditions as possible and were weighed once weekly. At the end of the injection period each animal was weighed and killed with coal gas. The testes, prostate, seminal vesicles, hypophysis, thyroids and adrenals were dissected out and at once weighed, paired organs or glands being weighed together. In removing the seminal vesicles care was taken not to express any of the contained secretion. In some experiments castrated animals were used, these being castrated through mid-ventral incisions under Avertin anaesthesia (0·65 ml. 2½ p.c. Avertin per 100 g. body weight intraperitoneally) about 1 week before the beginning of the injections. A list of the androgens studied, together with the number of injections and mean body weight changes in each group are given in Table I. For the sake of uniformity and to facilitate comparisons between the various groups, the group mean increase in body weight given in Table I is in each case that which had occurred after 28 days of injections irrespective of whether or not the experiment proceeded for a few more days. In two experiments, which only lasted for 27 days, the body weight increase over 27 days is given. A group of five guinea-pigs receiving daily injections of 2·0 mg. 17-methyltestosterone was injected for 34 days in connexion with another investigation. While therefore the weights of the organs removed at autopsy from these five animals were not comparable with those of the controls used in this study, their body weights at 28 days were available for use here. In respect of body growth over 28 days of injection, therefore, this group was combined with the group of three animals already referred to.

TABLE I

Injections	No. of guinea-pigs in group	No. of daily injections	Mean initial body weight g.	Mean body weight at end of experiment g.	Mean body weight increase g. (28 days)	Mean % body weight increase (28 days)
(a) 2 mg. androgen dissolved in 0·2 ml. sesame oil daily						
Sesame oil	8	31	188·6	302·1	100·4	55·0
Sesame oil	4 (castrated)	31	178·5	287·5	96·8	54·6
Δ_4-androstendione	5	32	162·4	290·4	119·2	74·7
Cis-androsterone	9	31	187·1	334·2	122·3	68·8
Δ_5-tr-dehydroandrosterone	5	31	179·2	322·0	113·4	62·6
Dihydrotestosterone	5	32	167·2	305·8	105·4	65·4
17-methyltestosterone	3	31	186·3	332·3	137·6*	79·0*
Testosterone	4	32	192·8	341·0	107·5	57·7
Testosterone propionate	5	32	177·8	320·0	111·8	62·4
Testosterone propionate	4 (castrated)	32	168·0	308·0	124·0	75·7
(b) 2 mg. androgen dissolved in 0·8 ml. sesame oil daily						
Sesame oil	9	28	192·1	316·5	124·4	68·1
Cis-androstandiol	5	28	164·4	276·8	112·4	69·5
Δ_5-tr-androstendiol	5	28	166·2	318·6	152·4	93·6
Δ_5-tr-androstendiol	5 (castrated)	28	182·4	277·0	94·6	52·0
Testosterone	5	27	176·4	313·0	136·6†	78·1
Testosterone propionate	5	27	176·6	307·2	130·6†	74·1

* These figures refer to a combined group of eight animals (see text). The mean initial body weight of this group was 174·0 g.

† 27 days.

RESULTS

The mean weights per 100 g. body weight of the paired testes, seminal vesicles, prostates and endocrine glands for each group are given in Table II, and subsequently referred to as "relative mean weights".

TABLE II. Mean weights in mg. per 100 g. body weight, of gonads, accessory reproductive organs and endocrine glands

Treatment	Testes	Prostate	Seminal vesicles	Hypophysis	Thyroid	Adrenals
(*a*) Daily oil volume 0·2 ml.						
Sesame oil	290·8	57·9†	116·3‡	3·3	12·5	45·4
Sesame oil (castrated)	—	26·9	19·0	3·7	13·1	51·4
Δ_4-androstendione	89·0	117·4	154·8	3·0*	14·3	52·1
Cis-androsterone	76·4	51·8	35·9	3·3	13·7	47·4
Δ_5-tr-dehydroandrosterone	234·0	71·3	142·2	3·6	12·1	44·5
Dihydrotestosterone	49·9	100·8	204·4	3·1	13·1	41·6
17-methyltestosterone	78·9	113·6	146·4	3·0	12·9	46·9
Testosterone	78·1	88·8	154·1	3·4	13·3	51·4
Testosterone propionate	74·9	99·1	306·7	3·6*	11·8	40·6
Testosterone propionate (castrated)	—	155·3	615·1	4·1	11·6	45·3
(*b*) Daily oil volume 0·8 ml.						
Sesame oil	230·4	56·9	65·6	3·2	12·0	51·9
Cis-androstandiol	37·3	63·8	144·4	3·1	10·9	53·3
Δ_5-tr-androstendiol	74·4	54·8	64·5	3·1	14·8	42·0
Δ_5-tr-androstendiol (castrated)	—	86·1	72·1	3·5	10·6	56·3
Testosterone	52·7	86·5	113·4	3·6	12·7	44·6
Testosterone propionate	71·4	125·6	290·6	3·0	12·7	41·2

* Mean of four. † Mean of five. ‡ Mean of six.

In the case of the relative mean weights of the testes, prostates and vesicles (Table II) and of the percentage increases in body weight during the first 28 days of each experiment (Table I), the significance of the difference between each mean and the corresponding control value was estimated by Fisher's t test [see Fisher, 1934]. The relative mean weights of the endocrine organs other than the testes, for the various groups, clearly differed so little from the controls that application of the above test was superfluous. Table III gives the values of P so obtained, where P = the probability that an effect at least as great as the observed effect should have arisen purely as an accident of random sampling. The commonly accepted criterion that two means differ significantly is that P shall be equal to, or less than, 1 in 20, i.e. $P \leqslant 0{\cdot}05$.

Weight of testes. The results in Table III show that all of the androgens studied with the exception of Δ_5-tr-dehydroandrosterone, caused, in the dosage given, a significant atrophy of the testes.

TABLE III

Treatment	Testes	Prostate	Seminal vesicles	Body weight increase (%)
(*a*) Daily oil volume 0·2 ml.				
Sesame oil (castrated)	—	**0·0048** ↓	0·806	0·973
Δ_4-androstendione	**0·0006** ↓	**0·0040** ↑	0·426	0·206
Cis-androsterone	**<0·0001** ↓	0·44	**0·026** ↓	0·277
Δ_5-tr-dehydroandrosterone	0·315	0·49	0·671	0·519
Dihydrotestosterone	**0·0001** ↓	**0·0194** ↑	0·128	0·477
17-methyltestosterone	**0·0001** ↓	**0·0008** ↑	0·619	**0·027** ↑
Testosterone	**0·0032** ↓	**0·014** ↑	0·488	0·858
Testosterone propionate	**0·0002** ↓	0·089	**0·023** ↑	0·603
Testosterone propionate (castrated)	—	**0·001** ↑	**0·0012** ↑	0·245
(*b*) Daily oil volume 0·8 ml.				
Cis-androstandiol	**<0·0001** ↓	0·468	**0·028** ↑	0·922
Δ_5-tr-androstendiol	**0·0006** ↓	0·784	0·96	0·109
Δ_5-tr-androstendiol (castrated)	—	**0·0166** ↑	0·807	0·305
Testosterone	**0·0002** ↓	**0·0246** ↑	0·0528	0·480
Testosterone propionate	**0·0012** ↓	**0·0004** ↑	**0·0004** ↑	0·696

Significant values of P (i.e. cases where $P \leqslant 0{\cdot}05$) are printed in heavy type.

↓ Against a mean signifies that the latter was smaller than the control value.

↑ Against a mean signifies that the latter exceeded the control value.

As regards the relative activities of the various androgens in causing testicular atrophy, nothing can be deduced from the relatively small differences between the various mean testes weights (Table II) since these do not differ significantly from one another. This is not surprising in view of the high-dosage and long-injection periods which probably more than sufficed to produce maximal effects.

Since Korenchevsky *et al.* [1937] were only able to demonstrate a slight decrease in testicular size due to androgen administration in mature males, it was of interest to determine whether administration of androgenic hormone to immature animals merely prevents growth of the immature testis or whether it causes any absolute regression. Twenty young male guinea-pigs of the usual body weight were used to elucidate this point. Sixteen were given twice-weekly subcutaneous injections of 3 mg. testosterone propionate dissolved in 0·2 ml. sesame oil, the other four being killed on the day of the first injection. Four of the sixteen injected animals were killed at weekly intervals, beginning 7 days after the first injection. The mean absolute weights of the paired testes for each group of four are plotted in Fig. 1.

Fig. 1 clearly shows that administration of testosterone propionate causes absolute regression of the testes in the immature guinea-pig. It will be noted that the testes began to grow again towards the end of this experiment. It is possible that this was due to the gradual fall in the dose of testosterone propionate per unit of body weight which resulted from the growth of the guinea-pigs.

Regarding the mechanism by which androgenic substances influence the immature testis it is probable that, as suggested by Moore & Price [1932], the suppression of the gonadotropic hormone secretion of the pituitary is involved. In a manner similar to that of these authors we have shown that testis atrophy does not result from administration of 2 mg. testosterone as propionate daily for 32 days to immature male guinea-pigs provided sufficient anterior lobe gonadotropic extract is injected at the same time (Table IV).

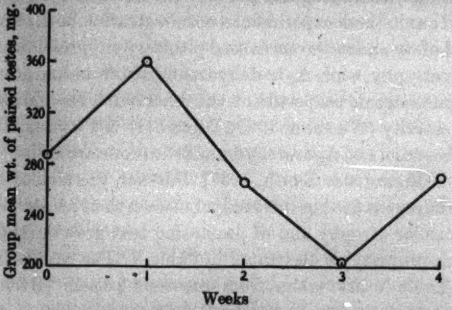

Fig. 1. Progressive changes in testis weight caused by administration of testosterone propionate.

TABLE IV. The effect of anterior pituitary extract in protecting the testes against the damage caused by administration of androgen

Treatment	No. of animals in group	Mean weight of testes at end of experiment mg.
2 mg. testosterone as propionate daily for 32 days	5	247·0
2 mg. testosterone as propionate daily for 32 days + 10 mg. horse anterior lobe extract AP61B daily	5	1581·8

It should be pointed out that though the results given in Table IV and similar ones obtained by Moore & Price [1932] are consistent with the theory that androgenic substances affect the testis by way of the pituitary, this type of experiment does not provide a critical test of this theory. Further evidence may be provided by the findings of the histological examination of the pituitaries after androgenic stimulation, which is being undertaken and also by further physiological experiments on hypophysectomized animals.

As far as it is possible from our results to draw conclusions as to the relative pituitary suppression potencies of any of the substances used by us, it may be said that the power of causing pituitary suppression appears to be correlated with androgenic potency rather than with gynaecogenic properties. Thus in these experiments, administration both of Δ_5-tr-androstendiol and of cis-androsterone caused pituitary suppression as evidenced by testicular atrophy, while Δ_5-tr-dehydroandrosterone had no effect. With regard to gynaecogenic properties on the other hand, cis-androsterone has little or no activity [Warren, 1935; Deanesly & Parkes, 1936], while Δ_5-tr-androstendiol and Δ_5-tr-dehydroandrosterone are active [Deanesly & Parkes, 1936; see also Koch, 1937]. Further, the lack of correlation, among the androgens used in this study, between their respective powers of causing testicular atrophy and of promoting teat growth (the latter is a gynaecogenic property) is illustrated in Table V. The results in the third column of Table V are taken from previous papers [Bottomley & Folley, 1938*a*, *b*] in which a study of the teats and mammary glands of the guinea-pigs used in the present work was reported.

TABLE V. Effect of various androgens on the teats and testes of male guinea-pigs

Androgen injected	Power of causing testicular atrophy	Teat growth promoting power
Cis-androstandiol	+	−
Δ_5-tr-androstendiol	+	+
Δ_4-androstendione	+	−
Cis-androsterone	+	−
Δ_5-tr-dehydroandrosterone	−	+
17-methyltestosterone	+	+
Testosterone	+	+

On the other hand, it is noteworthy that with respect to the power of producing pituitary suppression, the two male hormones found in male urine fall into the order of potency, cis-androsterone > Δ_5-tr-dehydroandrosterone which is the same as their relative order of potency on the basis of the capon comb test [see Koch, 1937].

The weight of the prostate. The results in the first two horizontal columns of Table II and the first horizontal column of Table III demonstrate clearly the well-known atrophy of the prostate which follows castration. From the remainder of the results the following conclusions emerge.

(1) Significant growth of the prostate over that of the controls occurred after administration, to normal guinea-pigs except where

otherwise stated, of Δ_4-androstendione, dihydrotestosterone, 17-methyltestosterone, testosterone at both dilutions, testosterone propionate in castrated guinea-pigs at the lower dilution and in intact ones at the higher dilution, and finally of Δ_5-tr-androstendiol in castrated guinea-pigs.

(2) No significant growth of the prostate over that of the controls was caused by the administration, to normal guinea-pigs except where otherwise stated, of Δ_5-tr-dehydroandrosterone, cis-androsterone, testosterone propionate at the lower dilution, cis-androstandiol, and Δ_5-tr-androstendiol.

(3) From the above it follows that under the conditions of these experiments, testosterone propionate was more active in causing growth of the prostate of the normal male guinea-pig when administered at the higher of the two dilutions used, particularly since the injection period in the latter case was 5 days shorter than in the case of the experiment at the lower dilution.

The effect of castration on the response of the immature guinea-pig prostate to testosterone propionate and Δ_5-tr-androstendiol will be considered later in conjunction with the effect of castration on the response of the seminal vesicles to these substances.

The weight of the seminal vesicles. Statistically significant increases in the relative mean weights of the seminal vesicles over those of the controls were observed in only four groups (see Table III), namely, those composed of intact guinea-pigs receiving testosterone propionate at both dilutions, castrated guinea-pigs receiving testosterone propionate and intact guinea-pigs receiving cis-androstandiol. Testosterone propionate and cis-androstandiol, therefore, appear to be the most active of the substancs studied as regards stimulation of the growth of the seminal vesicles in the intact guinea-pig under the condition of these experiments.

Table III shows that the mean relative weight of the seminal vesicles of the group receiving cis-androsterone was significantly less than that of the controls. This well illustrates the fact that the final sizes of the accessories of our intact animals were influenced by at least two factors: (1) the direct action of the injected hormone, and (2) the loss of the endocrine activity of the testes due to atrophy. In the case of the group receiving cis-androsterone the second influence was present and the first evidently almost absent, since the vesicles grew very much less than those of the control animals with normal testes. To take another example, in the group receiving Δ_5-tr-androstendiol, the mean relative prostate and vesicle weights were about equal to those of the controls. This at first sight suggests that this androgen had caused no growth of the accessories.

In view of the fact that the testes atrophied however, it is clear that as regards growth of the accessories, Δ_5-tr-androstendiol possessed sufficient activity to make up for the diminished endocrine activity of the testes.

It will be noted (see Tables II and III) that in some experiments the treatment caused a statistically significant increase in the mean relative weight of the prostate, while no statistically significant increase in the mean relative weight of the seminal vesicles could be demonstrated. This does not necessarily mean that the androgens used in these experiments stimulate prostate growth but have no action on the seminal vesicles, since in most of the experiments the coefficient of variation $\left(\frac{\sigma \times 100}{M}\right.$, where σ is the standard deviation of the mean $\left.M\right)$ of the seminal vesicle weights was considerably greater than that of the prostate weights. This also held for both control groups, and it is easy to see that in cases where the difference between the mean relative vesicle weights for the experimental and control groups was not very large, P would tend to be high.

There were six groups, namely those composed of animals receiving injections of Δ_4-androstendione, Δ_5-tr-dehydroandrosterone, dihydrotestosterone, 17-methyltestosterone and testosterone at both dilutions, in which though the mean seminal vesicle weights exceeded those of the controls, the level of significance of the difference was quite low. If, in any of these cases, differences of the order observed were in fact real, the relatively high scatter characteristic of vesicle weight data in these experiments would have necessitated, to demonstrate that the differences were statistically significant, the use of larger groups than we used.

From the foregoing it can be concluded that under our conditions fewer animals are required to show, at a satisfactory level of significance, that a given dose of androgen causes growth of the immature guinea-pig prostate as compared with controls than to do the same for the seminal vesicles. The prostate response may therefore be more useful for androgenic assay purposes than the seminal vesicle response. It is well known, of course, that the threshold for androgenic stimulation of the rat prostate is less than for the vesicles.

The effect of castration on the response of the prostate and seminal vesicles to androgens. The results given in Table II allow of comparisons between the weights of the prostate and seminal vesicles in normal as against castrated guinea-pigs after administration of both testosterone propionate and Δ_5-tr-androstendiol. In Table VI the mean relative

TABLE VI

	Mean weight of prostate, mg./100 g. body weight		Mean weight of seminal vesicles, mg./100 g. body weight	
Testosterone propionate (normal guinea-pigs)	99·1	(P=0·086)	306·7	(P=0·039)
Testosterone propionate (castrated guinea-pigs)	155·3		615·1	
Δ_5-tr-androstendiol (normal guinea-pigs)	54·8	(P=0·029)	64·5	(P=0·78)
Δ_5-tr-androstendiol (castrated guinea-pigs)	86·1		72·1	

weights of the prostates and seminal vesicles for the groups concerned in these comparisons are given, together with the relevant values of P.

In the case of the experiment with testosterone propionate the mean relative weight of the prostates of castrated guinea-pigs considerably exceeded that of similarly treated intact ones, P being slightly less than 9 in 100, while in the case of the vesicles, the mean relative weight of which for castrated guinea-pigs was about double that for normal animals, P was somewhat lower, namely, about 4 chances in 100. In the experiment with Δ_5-tr-androstendiol the mean relative weights of the prostates for the normal and castrated groups differed as above, but in this case P was only about 3 in 100. In this experiment the mean relative weights of the seminal vesicles for the normal and castrated groups were identical within experimental error.

Taken together, the above results strongly suggest that under the conditions of these experiments, androgenic substances have a greater effect on the accessory reproductive organs in the immature castrated animal than in the immature normal animal. This finding requires further study.

Effect of administration of androgens on body growth. Zondek [1936 *a*, *b*] has shown that treatment with oestrogens not only depresses the gonadotropic potency of the pituitary gland, but, under suitable conditions, also lowers the growth-promoting activity of the hypophysis. He found that prolonged administration of high doses of oestrogen to young rats inhibited their growth as compared with controls. Dwarfs so experimentally produced responded to anterior pituitary extracts by renewed growth.

In view of the analogy between the action of male and female hormones on the gonadotropic potency of the anterior lobe of the hypophysis, it might be expected that the growth-promoting activity of the pituitary would be depressed as a result of androgenic treatment just as happens after injections of oestrogenic hormone. It is, therefore, somewhat surprising to find on reference to Tables I and III, that none of the androgens studied by us, with the exception of 17-methyltestosterone, had any significant effect on body growth despite the high dosage given. In the one experiment in which a significant effect was observed, administration of 17-methyltestosterone caused not a decreased but, on the contrary, an increased growth rate as compared with the controls ($P=0{\cdot}027$). It is just possible that our treatment was not sufficiently prolonged for possible growth inhibition to manifest itself, though against this it may be noted that the effects observed by Zondek [1936*b*] became appreciable after 4 weeks' treatment.

The weights of the endocrine glands. Inspection of Table III makes it clear that in no experiment did the mean relative weights of the hypophysis, thyroid or adrenals differ significantly from those of the controls. Administration of oestrogenic hormones under suitable conditions causes enlargement (sometimes up to four times normal) of the hypophysis in both male and female rats [Cramer & Horning, 1936; Zondek, 1936*c*; McEuen *et al.* 1936; Leiby, 1933; Bacsich & Folley, 1938]. There is some disagreement as to the effect of oestrogen treatment on the weight of the thyroid gland. Leiby [1933] observed thyroid enlargement in ovariectomized rats after treatment with oestriol, while Bialet-Laprida [1933] and Bacsich & Folley [1938] found that the thyroids of rats which had received injections of oestrogenic hormone were lighter than those of controls. As regards the adrenals, Leiby [1933] observed adrenal enlargement in ovariectomized rats following treatment with oestrogenic hormone. It is, therefore, noteworthy that under the conditions of these experiments, administration of high daily doses of androgenic substances had no detectable effect on the weights of these endocrine glands.

DISCUSSION

These experiments show that androgenic substances have an action analogous to that of oestrogens on the gonad of the immature animal. This action appears to be connected with the androgenic properties of these substances rather than with any gynaecogenic properties they may possess. It is almost certain that androgenic substances affect the gonad by depressing the gonadotropic potency of the hypophysis just as oestrogens do. The analogy between the two classes of hormone appears to end there, however, since administration of androgens caused no retardation of growth in immature animals, even though high daily doses were given over periods of 4 weeks. Nor did the treatment cause the striking pituitary enlargement which almost invariably follows treatment with adequate doses of oestrogenic hormone. The thyrotropic and adrenotropic functions of the hypophysis were unaltered by androgenic treatment as far as can be judged from the weights of these endocrine glands. This, so far as the thyroid gland is concerned, is supported by histological examination, for which we are indebted to Dr A. S. Parkes. The thyroids of the androgen-injected animals showed no histological differences from those of the controls. It therefore seems clear that the action of androgens on the functions of the hypophysis though analogous to that of oestrogens so far as the pituitary gonadotropic function is concerned, is different in other respects. In this connexion it is of interest to note that according to Zondek [1936 *b*] the various pituitary functions are not simultaneously depressed by oestrogen treatment, and the gonadotropic function is the first to be suppressed.

SUMMARY

1. The effects of the administration of various crystalline androgenic substances on the weights of the testes, seminal vesicles, prostate, hypophysis, thyroid gland and adrenals in the young male guinea-pig have been studied.

2. Cis-androstandiol, Δ_5-tr-androstendiol, Δ_4-androstendione, cis-androsterone, dihydrotestosterone, 17-methyltestosterone, testosterone, and testosterone as propionate administered in daily doses of 2 mg. for about 30 days caused atrophy of the testes. Similar doses of Δ_5-tr-dehydroandrosterone did not cause testis atrophy.

3. Administration of 2·36 mg. testosterone propionate daily did not produce testicular atrophy if suitable doses of horse anterior lobe extract were given simultaneously.

4. Administration of high doses of testosterone propionate to immature males caused an absolute regression in testis weight.

5. The power of suppressing the gonadotropic activity of the pituitary as evidenced by testicular atrophy appears to be associated with androgenic properties rather than with gynaecogenic properties.

6. Under the conditions of these experiments, administration to intact guinea-pigs except where otherwise stated of Δ_4-androstendione, dihydrotestosterone, 17-methyltestosterone, testosterone (at both dilutions studied) and Δ_5-tr-androstendiol to castrated guinea-pigs caused significant growth of the prostate but not of the vesicles, as compared with the controls. The reverse was true for cis-androstandiol and for testosterone propionate injected at the lower dilution. Neither the pros-

tates nor the vesicles of guinea-pigs receiving injections of Δ_5-tr-dehydroandrosterone and of Δ_5-tr-androstendiol were significantly heavier than those of the controls, while testosterone propionate injected into intact guinea-pigs at the higher of the two dilutions studied and into castrated ones at the lower dilution, caused significant growth of both prostate and vesicles as compared with the controls. The prostates of guinea-pigs receiving injections of cis-androsterone were not significantly different in weight from those of the controls, though the vesicles were significantly lighter. The probable explanation for this is discussed.

7. Administration of testosterone propionate and Δ_5-tr-androstendiol had a greater effect on the accessories of castrated guinea-pigs than on those of intact ones.

8. None of the androgens caused significant changes in the weights of the hypophysis, thyroids or adrenals.

9. Of the androgens studied only 17-methyltestosterone affected bodily growth. The percentage increase in body weight of guinea-pigs injected with 17-methyltestosterone was significantly greater than that of the controls.

We are indebted to Dr A. S. Parkes, for the horse anterior lobe extract AP61B, and to Dr K. Miescher and Messrs Ciba, Ltd., for the androgens used in this work. Dr P. White kindly advised us regarding the statistical treatment of the data. Our thanks are also due to Mr J. McClemont for affording facilities for working with guinea-pigs and to Mr S. Watson for technical assistance.

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THE INFLUENCE OF THE GLYCOTROPIC (ANTI-INSULIN) FACTOR OF THE ANTERIOR HYPOPHYSIS ON THE INSULIN SENSITIVITY OF THE HYPOPHYSECTOMIZED RABBIT

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The abnormal sensitivity to the hypoglycaemic action of insulin which follows the removal of the hypophysis was demonstrated by Houssay & Magenta in 1925, but it is only within the last few years that the action of crude anterior pituitary extracts on insulin sensitivity has been investigated [Di Benedetto, 1933; Cope & Marks, 1934; Lucke, 1934]. Young [1936*a*], using normal rabbits as test animals, found that the "anti-insulin" or "glycotropic" [Young, 1936*b*] factor of the anterior pituitary gland was present in preparations of prolactin as normally obtained, but further evidence was presented to show that the glycotropic factor is not identical with prolactin, nor with the gonadotropic or thyrotropic hormones [Young, 1937, 1938]. This evidence was obtained by the use of intact rabbits as test animals, and the possibility had to be considered that a relatively purified pituitary substance, which is active in normal animals, may be ineffective in hypophysectomized animals [cf. Nelson & Gaunt, 1936; Gomez & Turner, 1936*b*]. The object of the present investigation was to determine whether or not preparations of the glycotropic factor free, or almost free, from gonadotropic and thyrotropic hormones could diminish the exaggerated sensitivity to the hypoglycaemic action of insulin shown by the hypophysectomized rabbit.

METHODS

Hypophysectomy in the rabbit. A procedure modified from that of White [1933] was used. In all instances the completeness of the removal of the pituitary gland was verified by histological examination of the contents of the *sella turcica* at post-mortem examination.

Pituitary extracts. Prolactin prepared at low temperature from fresh ox anterior lobe by the method described by Young [1938] (called prolactin-C for convenience) was used as a source of the glycotropic factor in most experiments, although in a number of instances prolactin prepared from commercial (B.D.H., Ltd.) acetone desiccated ox anterior pituitary gland by the method of Bates & Riddle [1935] was used. The prolactin-C contained a trace of substances with gonadotropic or thyrotropic activities [cf. Young, 1938], but the prolactin prepared by the Bates-Riddle method contained no trace of these activities detectable by our methods [cf. Young, 1938].

General procedure in experiments. If an intact rabbit is given two injections of prolactin-C, each equivalent to 2 g. of anterior lobe tissue, during a fast of 21 hr., the hypoglycaemic response to 2 units of crystalline insulin administered intravenously at the end of this time is entirely, or almost entirely, abolished [see Young, 1938]. A method of procedure similar to that employed with intact animals was adopted for experiments with the hypophysectomized rabbits, although a number of important modifications were required.

The danger of allowing a hypophysectomized animal to fast for anything more than a short period is well known, so a standard fasting period of 2½ hr. was adopted for the experiments on operated animals. It was found that 0·1 unit of intravenous crystalline insulin produced, in a hypophysectomized rabbit from which food had been withdrawn 2½ hr. previously, about the same fall of blood sugar as did 2 units in an intact rabbit fasting 21 hr. before the insulin injection (see Fig. 1). 0·1 unit was therefore used as the test dose of insulin for the operated rabbits. In a few instances the blood sugar of the hypophysectomized animals which had received 0·1 unit of insulin remained at a low level for an hour or more, and hypoglycæmic symptoms ultimately developed. As a precautionary measure, therefore, the control experiments on the hypophysectomized rabbits were usually terminated after 60 min. by the intravenous injection of glucose.

The procedure adopted was as follows. A control blood sugar (Hagedorn-Jensen method) response to 0·1 unit of intravenous crystalline insulin after 2½ hr. fasting was determined within 2 or 3 weeks of removal of the pituitary gland. About a week later a subcutaneous injection of pituitary extract was given at 5.30 p.m. and another similar injection at 9.30 a.m. on the following morning. Food was withdrawn at 11.30 a.m. and the test dose of insulin administered at 2 p.m.

For convenience of comparison, blood sugar values are expressed as a percentage of the initial value. The initial values are given in all cases and were never far from 100 mg./100 c.c.

The dose of prolactin necessary to diminish the glycaemic response to insulin in the hypophysectomized rabbit under the above conditions was about three times that required in control experiments with intact rabbits, and in most instances a total amount of prolactin-C, equivalent to about 13 gm. of fresh ox anterior lobe tissue, was given.

RESULTS

In Fig. 1 is given the average blood sugar response to 0·1 unit of insulin of seven normal (Curve A) and nine hypophysectomized (Curve B) rabbits fasting for 2½ hr. A typical blood sugar response to 2 units of intravenous crystalline insulin in the intact rabbit fasting 21 hr. (Curve C) is included for comparison. The results represented in Fig. 1 illustrate the substantial increase in insulin sensitivity which follows removal of the hypophysis from the rabbit, and indicates that the response to 0·1 unit of insulin after the pituitary gland has been removed is comparable with

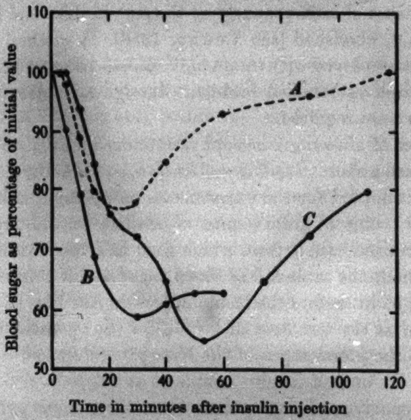

Fig. 1. Average blood sugar response for different groups of rabbits, to crystalline insulin administered intravenously. Curve A. Response of seven normal rabbits to 0·1 unit after a 2½ hr. fast. Curve B. Response of nine hypophysectomized rabbits to 0·1 unit after a 2½ hr. fast. Curve C. Response of a normal rabbit to 2 units after a 21 hr. fast. Initial blood-sugar levels: A, 113 mg./100 c.c.; B, 92 mg./100 c.c.; C, 110 mg./100 c.c.

that to 2 units in the intact animal under the conditions of these experiments.

Fig. 2 gives the average blood sugar response (Curve A) to 0·1 unit of insulin in seven hypophysectomized rabbits which had received two injections of prolactin-C equivalent to a total of about 13 g. of fresh ox anterior lobe tissue. Curve B (Fig. 2) is a typical response to 2 units of insulin of an intact rabbit which had received two injections of prolactin-C equivalent to a total of about 4 g. of fresh gland.

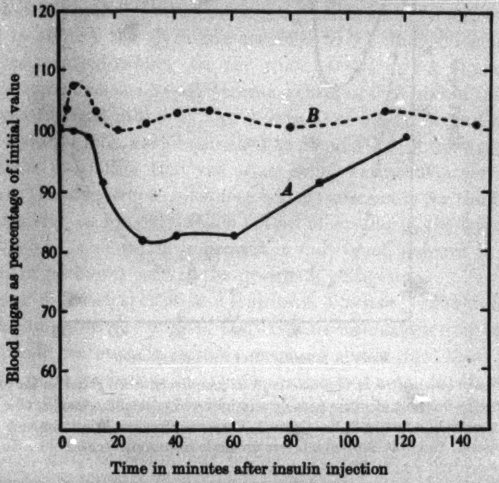

Fig. 2. Blood sugar response to intravenously administered crystalline insulin, of rabbits receiving two preliminary injections of prolactin-C. Curve A. Average response to 0·1 unit of seven hypophysectomized rabbits, fasting 2½ hr., which received two pituitary injections equivalent to a total of about 13 g. of fresh anterior lobe tissue. Curve B. Typical response to 2 units of an intact rabbit which had received two pituitary injections equivalent to a total of about 4 g. of fresh anterior lobe tissue. Initial blood-sugar levels: A, 89 mg./100 c.c.; B, 103 mg./100 c.c.

Prolactin prepared from commercial dried gland was also used. Fig. 3 gives the result of an experiment in which a rabbit received two injections, each of 125 mg. of a specimen of prolactin prepared according to the method of Bates & Riddle [1935] and the blood sugar response to 0·1 unit of insulin determined in the usual way (Curve A). The response of this rabbit to 0·1 unit before the prolactin injections is also given (Curve B).

The amount of this specimen of prolactin administered in this experiment was about three times that required almost to abolish the glycaemic response to 2 units of insulin in the intact rabbit.

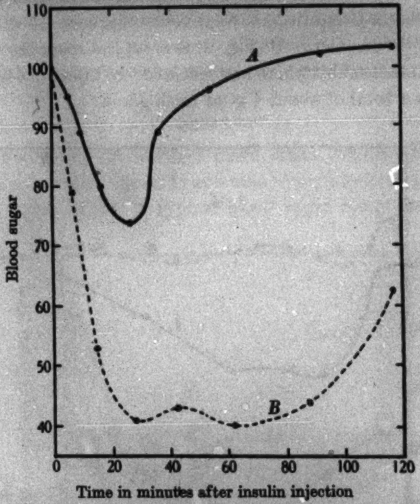

Fig. 3. Effect of two injections of prolactin from commercial dried gland on the blood sugar response to 0·1 unit of intravenously administered crystalline insulin of a hypophysectomized rabbit fasting 2¼ hr. Curve A. Response after two injections each of 125 mg. of prolactin. Curve B. Response before prolactin injections. Initial blood-sugar levels: A, 104 mg./100 c.c.; B, 92 mg./100 c.c.

DISCUSSION

The results show clearly that in hypophysectomized rabbits as well as in intact rabbits, the blood sugar response to insulin is diminished by the administration of preparations of prolactin containing the glycotropic factor, but the effectiveness of the glycotropic factor seems to be definitely decreased in the absence of the pituitary gland. The response to 0·1 unit of insulin in the hypophysectomized rabbit was not abolished by three times the dose of prolactin sufficient to eliminate the glycaemic response to 2 units of insulin in intact rabbits.

If the pituitary gland is normally secreting glycotropic substance in order to maintain the animal's normal degree of sensitivity to the hypoglycaemic action of insulin [Young, 1936*b*] it was to be expected that removal of this gland would increase the amount of exogenous glycotropic factor required to effect a given diminution in insulin sensitivity. Nevertheless, it is surprising to find that the large dose of prolactin used in these experiments was unable to do more than decrease the extent of the maximum fall of blood sugar in response to 0·1 unit of insulin to about one-half of what it would normally be in the hypophysectomized rabbit. A comparison of Curve A in Fig. 1 with Curve A in Figs. 2 and 3 suggests that the response of the prolactin-injected hypophysectomized rabbits to 0·1 unit is very similar to the response of the intact normal rabbit to a similar dose of insulin. It was not found possible with the extracts at our disposal to abolish the glycaemic response to 0·1 unit of insulin in the hypophysectomized rabbit. On the other hand, Cope & Marks [1934] were able to abolish the hypoglycaemic action of 0·25 unit of insulin in a hypophysectomized rabbit by two injections of a crude saline extract of fresh ox anterior lobe, each equivalent to about 1·5 g. of fresh gland. This suggests the possibility that the crude saline extract contains a factor other than the glycotropic substance which is necessary for the induction of insensitivity to hypoglycaemic action of insulin in the hypophysectomized rabbit, and which is present in only small amount in the preparations of prolactin used in the present investigation.

Nelson & Gaunt [1936] and Gomez & Turner [1936*b*] found that, although comparatively purified preparations of prolactin were capable of inducing milk secretion in suitably prepared guinea-pigs, these preparations were quite ineffective in hypophysectomized animals. On the other hand, if prolactin together with adrenotropic pituitary substance or cortin together with glucose was administered to hypophysectomized guinea-pigs, milk secretion was induced [Gomez & Turner]. It will obviously be of interest to determine whether or not the simultaneous administration of the glycotropic factor with comparatively purified pituitary substances of known physiological activity will be more effective in diminishing the hypoglycaemic action of insulin in the hypophysectomized rabbit, than were the preparations used in the present investigation.

Some authors believe that purified prolactin is incapable of causing growth of the crop-gland in hypophysectomized pigeons [Gomez & Turner, 1936*a*], but Schooley and others [1937] found that the crop-gland response of hypophysectomized pigeons was only about one-eighth that of intact birds. Thus the results of the present investigations do not provide the only instance of a diminution in sensitivity to the action of an anterior pituitary preparation as the result of hypophysectomy.

SUMMARY

1. The administration to hypophysectomized rabbits of preparations of prolactin containing the glycotropic factor of the anterior pituitary lobe, but almost free from the gonadotropic and thyrotropic factors, resulted in a substantial diminution of the hypoglycaemic action of 0·1 unit of insulin.

2. The preparations of the glycotropic factor used were considerably less effective in hypophysectomized rabbits than in normal rabbits. The possible reasons for this are discussed.

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UNDERNUTRITION AND LIVER FAT

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In the course of earlier investigations on the liver fat of white rats it was observed that the use of certain diets, with or without the addition of choline, usually resulted in a considerable loss of weight during the experimental period. Results, showing that this factor, within the limits encountered in the experiments referred to above, does not affect the interpretation of the findings in the choline experiments, have been published previously [Best & Huntsman, 1935; Best *et al.* 1935]. Furthermore, a diet low in lipotropic factors has now been devised which usually produces a gain in weight for at least a period of 3 weeks. When choline is also supplied [MacLean *et al.* 1937] the gain is maintained for longer periods. We became interested, however, in the effect of undernutrition on liver fat and are reporting the results of further studies which extend our previous observations.

Methods

White rats of the Wistar strain, approximately 200 g. in weight unless otherwise specified, were supplied from the Connaught Laboratories' colony for this investigation. The care of the animals has been described previously. Fat estimations were made by the direct saponification procedure. Fatty livers were produced by feeding a diet low in choline, which consisted of the following constituents: meat powder [MacLean *et al.*] 10 % by weight, beef dripping 40 %, commercial sucrose 43 %, salt mixture (McCollum's No. 185) 5 %, agar 2 % and adequate amounts of vitamins A, D and crystalline B_1 (Merck). One gram of this diet provided 5·7 cal. This diet was given for 21–24 days, i.e. the preliminary period referred to throughout the experiments.

The term "body fat" in this paper refers to the total fat remaining after the liver and gastro-intestinal tract have been removed. The fat values given in the tables are the average figures obtained from twelve to fifteen animals. The liver fat is expressed as grams per 100 g. of initial weight of rat.

EXPERIMENTAL RESULTS AND COMMENTS

Choline and the fatty liver of starvation

Mottram [1909], in guinea-pigs and rabbits, Dible [1932], in rats, Dible & Libman [1934], in rabbits, have reported that an infiltration of fat into the liver occurs during a short period of fasting (see also Dittmar [1933]; Wetzel *et al.* [1936] and Deuel *et al.* [1937]). This result is easily confirmed using guinea-pigs, rabbits or mice, but in rats we have found it difficult to obtain consistent results. The rats may show an increase in the percentage of fat in the liver and at the same time a diminution in the total amount. This apparent increase in percentage may be caused by shrinkage of liver due to loss of glycogen and water from the tissue. In some cases an increase in the percentage and also in the total amount of fat is observed. Reference to an experiment conducted by Mrs Mawson and one of us (C. H. B.) has been made previously [Best, 1934]. In this experiment, in which a large group of rats was used, an increase in the percentage of fat in the liver was found in the control series during 48 hr. fasting, but no increase was noted in those which had received huge doses of choline for a prolonged period *before* fasting and *also* during the period of starvation. When choline was given only during the period of fasting no effect was observed. The average fat content of the livers of normal rats in our experience is approximately 3·1%. In a recent experiment (Table I (1)) on twenty male rats the average value after 48 hr. starvation was 3·9%. In a similar series receiving approximately 70 mg. of choline in agar daily during the fast the average value was 3·7%. There was no indication of a direct relationship between body and liver fat in individual animals when all the above results were taken into account.

In a series of experiments in which large female rats with an average weight of 300 g. and with exceptionally large stores of body fat were used, moderately fatty livers were seen after 2 or 3 days' fasting. There was considerable individual variation as shown in Table I (2). In another experiment heavy male rats with an average weight of 400 g. and heavy female rats of 300 g. average weight were divided into groups. All the animals were starved and half of them received choline. The results shown in Table I (2) and (3) suggest that the livers of female rats deposit more fat during fasting than male rats. In both groups of animals there were large reserves of depot fat. In the first series of female rats it

TABLE I

					Liver fat		Body fat	
Experiment	Duration of experiment days	Av. daily caloric intake per 200 g. rat	Av. change in weight %	Liver % of initial body weight	per 100 g. rat (calc.) g.	%	Total fat g.	%
(1) Fasting male rats	2	0	−11	2·8	0·11	3·9 (3·2–4·8)	7·9	6·0
Choline daily	2	0	−11	2·8	0·10	3·7 (3·3–4·2)	7·4	5·5
(2) Fasting female rats	1	0	− 5	2·7	0·13	5·0 (4·4–5·7)	31·0	12·8
	2	0	−11	2·3	0·14	6·2 (5·3–7·9)	27·8	12·5
	3	0	−13	2·3	0·16	7·1 (5·5–9·1)	32·0	12·9
Fasting female rats, choline daily	1	0	− 4	2·5	0·12	5·4 (5·1–6·1)	40·1	14·5
	2	0	−11	2·3	0·12	5·2 (4·1–5·7)	24·0	10·4
	3	0	−12	2·0	0·11	5·3 (4·3–6·1)	26·3	11·6
(3) Fasting male rats	2	0	− 9	2·2	0·11	5·0 (4·4–6·2)	—	—
	3	0	−10	2·0	0·09	4·5 (3·7–6·2)	—	—
	4	0	−14	1·9	0·09	4·7 (3·9–5·5)	—	—
	5	0	−16	2·0	0·08	3·9 (3·7–4·2)	—	—
Fasting male rats, choline daily	2	0	− 9	2·1	0·09	4·5 (3·8–5·8)	—	—
	3	0	−10	2·0	0·09	4·7 (4·0–6·0)	—	—
	4	0	−14	2·0	0·08	4·2 (3·5–5·2)	—	—
	5	0	−11	2·2	0·09	4·4 (3·5–5·2)	—	—
Fasting female rats	3	0	−14	2·2	0·12	5·7 (5·1–6·8)	—	—
	4	0	−16	2·1	0·13	6·1 (3·8–9·7)	—	—
	5	0	−12	2·4	0·17	6·9 (5·1–8·6)	—	—
Fasting female rats, choline daily	3	0	−14	2·0	0·10	4·9 (4·2–6·2)	—	—
	4	0	−15	2·2	0·12	5·5 (4·3–6·5)	—	—
	5	0	−14	1·9	0·12	6·3 (4·2–9·0)	—	—
(4) Low choline diet	21	20	−25	3·1	0·18	8·3	—	—
	21	42	− 5	4·0	0·90	22·5	—	—
	21	60	+ 1	4·6	0·86	18·7	—	—

appeared possible that choline might have exerted a slight effect, but in the second series there was certainly no significant difference. The individual values varied widely. Unfortunately, sufficient animals of this type were not available to permit adequate exploration of the problem.

In collaboration with Dr James Campbell the action of choline has been tested on guinea-pigs, rabbits and mice. Attempts have been made to give as much choline as possible to the animals during the period of starvation by adding it to agar and to the drinking water. However, in spite of the administration of amounts which would be more than adequate to prevent the deposition of fat in the livers of rats or mice produced by a diet rich in fat and poor in choline, we failed to note any indication that choline was effective during 48 hr. of fasting.

The possibility that the fatty liver of starvation is produced by a deficiency of choline rather than by the absence of food can therefore be excluded in experiments of this type. It may also be pointed out that Best & Campbell [1936] were unable to demonstrate any effect of choline in preventing the fatty liver produced by injection of certain extracts of the anterior pituitary gland. It will be noted later, however, that when the livers of rats contain appreciable amounts of fat, an effect of choline during a subsequent period of starvation may be elicited.

The effect on liver fat of different caloric intakes

This investigation was made on forty-five normal male rats which were divided into three groups all of which received the diet low in choline. The first group received 20 cal. per 200 g. rat daily for 21 days, the second group 42 cal., and the third group 60 cal. daily. The results show that the total liver fat of the rats in the first group had increased very slightly and that the animals had lost 25% of their body weight (Table I (4)). Although the animals of the second group lost 5% of their body weight, there was no significant difference in the total amount of liver fat between this group and the third series of rats which had gained slightly in weight. This observation confirms our previous findings, i.e. that slight losses in weight have no effect on the deposition of liver fat in rats receiving a diet low in choline.

Undernutrition and the disappearance of liver fat

In the next series of experiments we investigated the effect on liver fat when animals with fatty livers were placed on diets of various caloric intakes.

(*a*) In the first experiment the animals were fasted. The effect of starvation on the disappearance of fat from livers which have been made fatty by feeding a high-fat diet has been described by Best & Huntsman. They observed a rapid fall in the liver fatty acids and a corresponding decrease in the body fats. The effect of choline was not definite. In a second unpublished experiment, however, these authors found a definite effect of choline. This experiment has been repeated and the results are shown in Table II (1). Fatty livers were produced in 100 male rats in the customary way. The liver and body fats were determined on fifteen control animals. The remainder were divided into two groups. All the animals received agar, but half of them were given 50 mg. of choline daily as an aqueous solution mixed with the agar. No trace of choline or other lipotropic factors has been found in the agar used. In the animals

TABLE II

Experiment	Duration of experiment days	Av. daily caloric intake per 200 g. rat	Av. change in weight %	Liver % of initial body weight	Liver fat		Body fat	
					per 100 g. rat (calc.) g.	%	Total fat g.	p.c.
(1) Preliminary period: Low choline diet. Control	24	56	+ 8	5·1	0·96	18·9	20·0	12·5
Experimental period: Fasting No choline	2	0	– 6	4·3	0·94	21·9	15·0	9·9
	3	0	– 11	3·9	0·67	17·2	12·5	8·9
	5	0	– 19	2·8	0·31	11·0	6·3	5·2
Fasting 50 mg. of choline daily	2	0	– 6	3·7	0·56	15·1	11·6	8·3
	3	0	– 12	3·5	0·47	13·6	11·4	9·0
	5	0	– 19	2·8	0·23	8·4	8·4	7·4
(2) Preliminary period: Low choline diet. Control	22	57	+ 1	4·3	0·75	17·4	26·2	13·5
Experimental period: (*a*) Same diet	7	11	– 13	3·0	0·43	14·2	—	—
	14	11	– 26	2·2	0·13	6·1	—	—
	21	10	– 42	1·2	0·05	3·9	1·9	1·7
(*b*) Same diet	7	21	– 9	3·4	0·51	15·1	—	—
	14	21	– 16	3·5	0·67	19·3	—	—
	21	21	– 29	3·4	0·68	19·9	10·0	6·7
(*c*) Same diet	7	37	– 2	4·4	0·80	18·3	—	—
	14	36	– 6	4·8	1·02	21·2	—	—
	21	37	– 11	4·3	0·98	22·9	16·4	9·9
	40	37	– 15	4·6	1·43	31·2	14·4	9·2
	78	36	– 22	4·0	1·14	28·4	11·5	8·3
(3) Preliminary period: Low choline diet (restricted). Control	21	34	– 16	3·2	0·29	9·2	13·6	7·8
Experimental period: Same diet	7	26	– 2	3·4	0·61	17·8	13·3	8·8
	14	26	– 5	3·3	0·69	21·0	—	—
	21	26	– 8	3·7	0·81	21·8	11·1	7·5

Table II (*cont.*)

Experiment	Duration of experiment days	Av. daily caloric intake per 200 g. rat	Av. change in weight %	Liver % of initial body weight	Liver fat		Body fat	
					per 100 g. rat (calc.) g.	%	Total fat g.	%
(4) Preliminary period: Low choline diet. Control	22	62	+ 6	4·7	0·82	17·3	18·8	11·9
Experimental period:	8	60	—	4·5	0·83	18·4	—	—
(*a*) Same diet	14	61	+ 2	4·7	1·29	27·4	—	—
	22	60	—	5·2	1·50	28·9	—	—
(*b*) Same diet	5	31	− 4	3·2	0·45	14·1	13·6	8·6
	8	30	− 7	3·2	0·39	12·1	—	—
	14	30	−10	2·4	0·28	11·6	—	—
	22	32	−22	2·3	0·22	9·5	4·8	3·7
(5) Preliminary period: Low choline diet. Control	21	64	+ 7	4·6	0·87	18·6	24·4	13·9
Experimental period:	7	20	− 8	3·0	0·41	13·6	—	—
(*a*) Same diet	14	20	−18	2·8	0·45	15·9	8·0	5·9
	21	20	−18	2·7	0·49	17·7	—	—
	28	20	−28	2·3	0·35	15·2	4·8	4·0
(*b*) Same diet	7	30	− 4	3·4	0·61	18·0	—	—
	14	30	−10	3·9	0·97	24·9	13·5	9·0
	21	30	−13	3·5	0·90	25·7	—	—
	28	30	−16	3·4	0·69	20·2	12·1	8·2
(6) Preliminary period: Low choline diet (restricted). Control	21	34	−10	3·1	0·49	15·7	11·5	7·3
Experimental period:	7	20	− 9	2·5	0·41	16·1	—	—
(*a*) Same diet	14	20	−17	2·5	0·37	14·8	4·7	3·8
	21	20	−30	2·2	0·32	14·5	—	—
	23	20	−33	1·7	0·16	5·2	2·4	2·6
(*b*) Same diet	7	30	− 4	3·5	0·80	23·0	—	—
	14	30	− 8	3·3	0·82	24·7	9·2	6·8
	21	30	−14	2·8	0·52	18·6	—	—
	28	30	−14	2·7	0·50	18·4	9·7	7·8

which were starved without choline there appeared to be a definite increase in the percentage of liver fat after 48 hr., but actually the total amount of liver fat remained approximately constant. This increase in percentage may be due in part to loss of glycogen and water with resultant shrinkage of the liver. In the series which received choline there was a definite decrease both in the percentage and total liver fat. Subsequently, in those which were starved without choline the liver fat decreased but the rate was not as rapid as in those which received choline.

(*b*) In the next series of experiments the effect of different degrees of undernutrition was studied on a group of rats with fatty livers. Fatty livers were produced in 144 male rats. After 22 days on the low-choline diet the liver fat of fifteen control animals was 17·4 %, the body fat was 13·5 % and the animals had all gained slightly during this preliminary period. The remaining rats were divided into three groups and placed on the same low-choline diet. The first group received an average of 11 cal. daily per 200 g. rat, the second 21 cal. and the third 37 cal. Rats from each group were killed at 7, 14 and 21 days, and in the third group the period was extended to 40 and 78 days. A summary of the results is given in Table II (2) and shown graphically for 40 days in Fig. 1. In the first group in which the intake was only 11 cal. per day, there was a steady decrease in liver fat. The animals became very emaciated and at the end of 21 days they had lost 42 % of their initial weight. They could not have survived much longer as their body fat had fallen to a very low level. With an intake of 21 cal. per day there was a transient decrease in liver fat in 7 days and this decrease was followed by a rise which appeared to reach a steady value in 21 days. The rate of weight loss was much less than in the previous group and at the end of the experiment a considerable amount of body fat remained. In the third group of rats which received 37 cal. per day, the rate of weight loss was very slow when compared with the other groups in which the caloric intake was more restricted. In 78 days the rats had only lost 22 % of

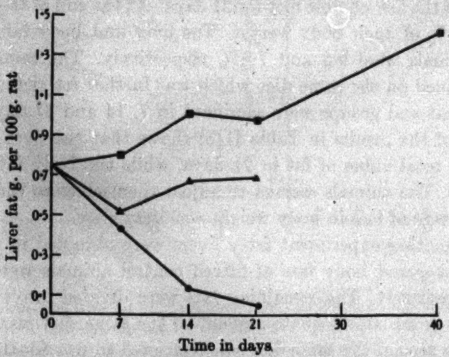

Fig. 1. The effect on liver fat of various caloric intakes of the diet low in choline (Table II (2)). ■—■ 36·6 cal. daily per 200 g. rat. ▲—▲ 21 cal. daily per 200 g. rat. ●—● 10·6 cal. daily per 200 g. rat.

their initial weight. The deposition of liver fat continued to increase for 40 days, and even at the end of the 78 days the total liver fat was much greater than at the end of the preliminary period, while the disappearance of body fat was very much slower than in the previous groups.

(*c*) The next experiment was planned to see what effect a lower level of liver and body fat at the end of the preliminary period would have on the liver fat in the experimental period during which the caloric intake was further reduced. In order to obtain less deposition of body fat in the preliminary period the rats received the equivalent of only 34 cal. per 200 g. rat of the low-choline diet for 21 days. At the end of this time they had lost 16% of their body weight. The liver and body fats of fifteen control animals were 9·2 and 7·8% respectively. The remaining rats were continued on the same diet which was further restricted to 26 cal. per 200 g. rat and groups were sacrificed in 7, 14 and 21 days. An examination of the results in Table II(3) shows that the liver more than doubled its total value of fat in 21 days, while the body fat decreased very slowly. The animals seemed to adjust themselves to the restricted diet as the rate of loss in body weight was very slow.

(*d*) In another experiment fatty livers were obtained in 120 female rats. The liver and body fats of fifteen control animals were 17·3 and 11·9% respectively. The remaining rats were divided into two groups. In the first group an adequate amount of the same diet was continued while in the second the same diet was restricted to provide 31 cal. daily per 200 g. rat. The summary of the results in Table II(4) shows that there is a continued deposition of liver fat in the rats which received the adequate diet. In the group in which the caloric intake was restricted the liver fat and body fat have shown a steady decrease. These results are not in quantitative agreement with the ones reported in Table II(5) (*b*) of this same series, but it could not be expected that the critical level of caloric intake producing a rise or fall of liver fat would be the same in different groups of animals.

In another experiment the effect of an adequate or a restricted intake in the preliminary period on the curve of liver fat during the experimental period was studied. 270 male rats were placed on the diet low in choline: 135 of the animals received 64 cal. and the remainder 34 cal. per 200 g. rat for 21 days. At the end of this preliminary period the liver and body fats were much higher in the first than in the second group. Both groups were continued on the same diet but the intake was reduced to 20 cal. per 200 g. in half the animals of each group. The remainder received 30 cal.

per 200 g. Groups were killed each week for 4 weeks. The results are shown in Table II (5) and (6), and the changes illustrated graphically in Figs. 2 and 3.

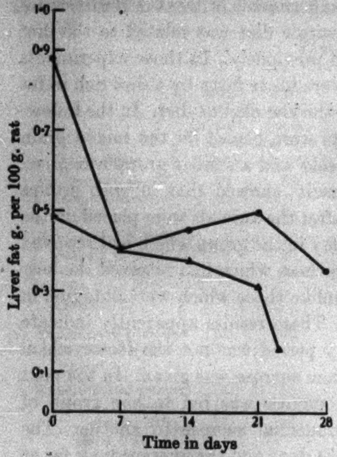

Fig. 2.

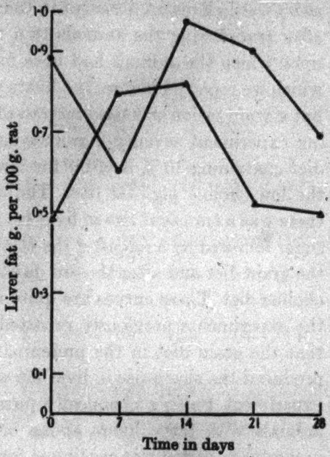

Fig. 3.

Fig. 2. The effect on liver fat of 20 calories of the diet low in choline daily per 200 g. rat (Table II (5) and (6)). ●—● 64 cal. daily per 200 g. rat in the preliminary period. ▲—▲ 34 cal. daily per 200 g. rat in the preliminary period.

Fig. 3. The effect on liver fat of 30 calories of the diet low in choline daily per 200 g. rat (Table II (5) and (6)). ●—● 64 cal. daily per 200 g. rat in the preliminary period. ▲—▲ 34 cal. daily per 200 g. rat in the preliminary period.

High-carbohydrate diets and liver fat

It has been reported from these laboratories [Best & Huntsman] that, when a diet composed entirely of sucrose is fed to rats whose livers had previously been made fatty by a grain diet rich in fat, a rapid and extensive further rise in liver fat may occur. This is not the result of one experiment only but of four. More recently we have attempted to extend these experiments using as far as possible the same procedure. In the first experiment seventy-five male rats were fed for 3 weeks on the low-choline and high-fat diet. At the end of this period liver and body fats were determined on fifteen rats. The rest of the animals were placed on a commercial sucrose diet and groups were killed at intervals of 3, 6, 9 and 13 days. A steady *decrease* in liver fat was observed, but, to conserve space, these values and those of the next two experiments are not given in the tables.

In the next experiment we decided to test the possibility that the *sharp rise* in liver fat observed in the experiments of Best & Huntsman after transferring the animals to a sucrose diet was related to the diet upon which the animals had been fed previously. In those experiments which we reported before, the livers were made fatty by a diet rich in fat but a grain ration was used instead of the low-choline diet. In the following experiment seventy-five male rats were placed on the mixed grain diet containing 40% beef fat for 3 weeks and a similar group was given the low-choline high-fat diet. The results showed that in both groups there was a transient rise in liver fat after the animals were placed on the sugar followed by a *fall* after the 6th day in the group which had received the grain diet and after the 3rd day in those which had received the low-choline diet. These curves are quite unlike those which were obtained in the experiments previously reported. These results apparently indicate that the grain diet in the preliminary period was not the factor which produced the sharp rise in liver fat when sucrose was given. In the next experiment Baker's chemically pure sucrose was fed to one group of animals with fatty livers and a commercial sucrose to another. The preliminary period was continued for 54 days and the average liver fat at this time was 25·6%. In both cases when the animals were placed upon the carbohydrate diet there was a slight rise in liver fat; with purified sucrose the peak was reached in 3 days while with the commercial sucrose the maximum point was somewhat later. After the crest of the curve was reached the liver fat fell off in both groups. In the next experiment (Table III (1)) after the livers had been made fatty on the diet low in choline, the animals were transferred to Baker's sucrose to which vitamin B_1 and Cellu flour had been added in the amounts indicated in the table. The results show that there was very little change in liver fat over a period of 13 days. It will be noted, however, that there was a slight decrease until the 9th day and then an increase on the 13th. As will be seen later, a delayed increase of this type may be significant.

In the next experiment (Table III (2)) fatty livers were produced in 144 female rats, and after 21 days liver and body fats were estimated on fifteen animals. The remainder were transferred to a diet containing 90% sucrose, 5% agar and 5% salt mixture. Vitamins A, D and B_1 were provided. In one group the animals were allowed to eat as much of this diet as they would. In two other groups a "paired feeding" experiment was conducted. In one of these groups no vitamin B_1 was provided.

The results show that in all three cases there was a preliminary fall in liver fat when the animals were transferred to the sucrose diet, but that in the first group after the 8th day the liver fat began to rise and by the 21st day had reached a level appreciably higher than at the start. In

TABLE III

Experiment	Duration of experiment days	Av. daily caloric intake per 200 g. rat	Av. change in weight %	Liver % of initial body weight	Liver fat: per 100 g. rat (calc.) g.	Liver fat: %	Body fat: Total fat g.	Body fat: %
(1) Preliminary period:	27	56	+ 7	4·2	0·68	16·2	19·1	12·7
Low choline diet.								
Control								
Experimental period:	3	30	− 5	4·4	0·57	13·0	16·1	11·5
Baker's sucrose	9	28	−11	4·3	0·47	11·1	—	—
C.P. 80 p.c.	13	35	−17	3·4	0·53	15·3	8·1	6·8
Cellu flour 20%								
Vitamin B_1								
(2) Preliminary period:	21	63	+ 3	4·0	0·61	15·3	32·4	15·8
Low choline diet.								
Control								
Experimental period:	8	36	− 3	4·1	0·52	12·9	—	—
(*a*) Commercial	15	34	−16	4·1	0·55	13·7	—	—
sucrose 90%	21	32	−22	3·7	0·70	19·0	10·6	7·3
Agar 5%								
Salt 5%								
Vitamins A, D, B_1								
(*b*) Same as (*a*)	8	33	−14	3·2	0·31	9·8	—	—
without B_1	15	24	−21	2·5	0·15	6·0	—	—
	21	20	−30	2·3	0·17	7·6	4·0	3·2
(*c*) Same as (*a*)	8	30	−11	3·3	0·38	11·7	—	—
paired feeding	15	24	−18	3·2	0·34	10·7	—	—
with (*b*)	21	20	−25	2·4	0·28	11·5	9·1	5·8
(3) Preliminary period:	21	57	+ 3	4·8	0·72	15·0	16·8	10·9
Low choline diet.								
Control								
Experimental period:	3	42	− 3	4·3	0·60	13·9	18·8	11·2
Commercial	7	44	−11	4·8	0·60	12·5	17·0	11·2
sucrose 98%	14	40	−14	4·3	0·58	13·4	15·7	10·4
Agar 1%	21	41	−22	4·3	0·64	15·1	—	—
Salt 1%	28	36	−26	4·6	0·85	18·4	11·4	9·0
Vitamins A, D, B_1	35	33	−31	3·8	0·67	17·6	9·5	7·8
(4) Preliminary period:	21	34	−16	3·2	0·29	9·2	13·6	7·8
Low choline diet								
(restricted).								
Control								
Experimental period:	3	44	—	4·2	0·49	11·7	—	—
Commercial	7	44	− 4	4·1	0·41	10·0	—	—
sucrose 98%	21	36	−15	3·8	0·59	15·7	12·6	9·2
Agar 1%	28	36	−21	3·1	0·41	13·1	—	—
Salt 1%	35	34	−25	3·3	0·51	15·2	—	—
Vitamins A, D, B_1	43	33	−31	2·9	0·45	15·4	—	—
	49	31	−27	3·1	0·58	18·7	7·7	6·6

TABLE III (*cont.*)

Experiment	Duration of experiment days	Av. daily caloric intake per 200 g. rat	Av. change in weight %	Liver % of initial body weight	Liver fat		Body fat	
					per 100 g. wt. (calc.) g.	%	Total fat g.	%
(5) Preliminary period: Low choline diet (restricted). Control	21	32	− 9	3·5	0·47	13·2	15·8	9·4
Experimental period:	3	40	− 2	4·3	0·68	14·7	—	—
(*a*) Commercial	6	36	− 6	3·7	0·48	12·0	12·6	7·8
sucrose	10	32	− 9	3·1	0·31	10·1	—	—
	21	23	−25	2·7	0·36	13·3	4·1	3·2
(*b*) Commercial	3	40	− 1	4·1	0·55	13·1	—	—
sucrose 98 %	6	42	− 5	3·8	0·34	8·8	13·3	8·3
Agar 1 %	10	42	−12	4·2	0·59	14·0	—	—
Salt 1 % Vitamins A, D, B_1	21	35	−15	3·7	0·55	14·9	12·0	8·5
(6) Preliminary period: Low choline diet. Control	21	62	+10	3·8	0·59	15·5	26·1	16·4
Experimental period:	5	39	− 7	4·3	0·70	16·3	—	—
(*a*) Commercial	11	31	−15	3·8	0·73	19·2	15·6	9·7
sucrose	18	24	−21	3·0	0·37	12·3	—	—
(*b*) Commercial	5	38	− 7	4·6	0·84	18·2	—	—
sucrose 98 %	11	37	−13	4·8	0·92	19·2	11·8	12·3
Agar 1 % Salt 1 % Vitamins A, D, B_1	18	29	−20	4·4	0·84	19·0	—	—

both the other groups there was no later rise but the animals receiving vitamin B_1 maintained a definitely higher liver fat than those without this vitamin. These results support those published by McHenry [1937].

In the next experiment (Table III (3)) it was decided in view of the late rise in liver fat in one group of animals in the last series that a prolonged period of sucrose feeding should be carried out. The preliminary part of the experiment was the same as that described previously. In this experiment when the animals were placed upon a sucrose diet which contained vitamins A, D and B_1, there was a slight preliminary fall in liver fat, then a period in which there was no change. Here again, however, after the 14th day the fat began to rise. On the 28th day it reached a level appreciably greater than at the start of the sucrose feeding. In this experiment there was subsequently, between the 28th and 35th day, a falling off in the fat content of the liver.

Since we were not able to secure the rapid and extensive rise noted in previously published experiments, we decided in the next experiment

(Table III (4)) to place the animals during the period in which their livers were getting fatty on a *diet of low caloric intake.* After 21 days on this diet low in choline the animals had lost approximately 16% of their body weight. They were then placed upon the sucrose and vitamin ration and groups were killed at intervals up to 49 days. In this experiment there was no preliminary fall in the liver fat when the animals were placed on the sucrose diet. The slight rise appeared to be definite. This rise after a slight fall continued, and 21 days from the start of the sucrose feeding a rather high level of liver fat had been obtained. This subsequently decreased slightly on the 28th day, but on the 35th and 49th days there was again a rise. These changes can be seen clearly in Fig. 4.

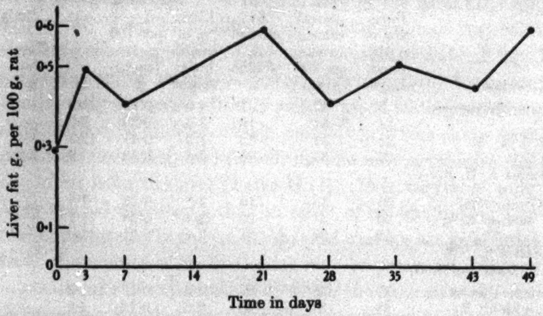

Fig. 4. The effect on liver fat of a diet containing commercial sucrose, etc. (Table III (4)).

Another somewhat similar experiment was carried out. 135 rats received daily 32 cal. per 200 g. provided by the diet low in choline for 21 days (Table III (5) (*a*) and (*b*)). The animals were then divided into two groups. One group received sucrose only while the other was given in addition vitamins A, D and B_1. The results are similar to those described previously.

In the last experiment of this type (Table III (6)) the rats received adequate amounts of the diet low in choline during the preliminary period. In the experimental period the animals were divided into two groups both of which received sucrose and one group the vitamin mixture. In both groups there was an initial rise in liver fat which persisted for 11 days. By the 18th day, however, there was a marked decrease in the total and percentage liver fat of the first group, while in the group which received the vitamin mixture the decrease was very slight.

The effect of feeding sucrose on the liver fat of normal rats

In the next series of experiments the effect of feeding sucrose on the liver fat of normal rats was studied. Best & Huntsman had previously reported a rise in liver fat when normal rats were placed on a diet consisting exclusively of sucrose. These results have been confirmed and extended in the present work. In the first experiment forty-eight female rats were placed on a diet consisting of sucrose 90%, agar 5%, salt mixture 5% with vitamins A, D and B_1. Twelve animals were killed at 7, 14, 22 and 27 days. The results (Table IV (1)) show a very definite rise in liver fat which reaches the level of approximately 14% on the 27th day. In the next experiment of this series (Table IV (2)) 180 male rats were divided into four groups of forty-five rats each. Fifteen animals from each group were killed at intervals of 20, 28 and 35 days. In the first group the animals were allowed to eat all the sucrose and vitamin ration which they would take. The results show a definite and steady rise in liver fat. In the second series the same ration was provided but no vitamin B_1 was given. In this series there was a very slight rise in liver fat. In the third group the food intake was the same as that of the animals in the second group but vitamin B_1 was provided. In this group the increase of liver fat was definitely greater than in the second group.

TABLE IV

Experiment	Duration of experiment days	Av. daily caloric intake per 200 g. rat	Av. change in weight %	Liver % of initial body weight	Liver fat		Body fat	
					per 100 g. rat (calc.) g.	%	Total fat g.	%
(1) Commercial sucrose	7	37	− 7	4·0	0·19	4·8	—	—
90 %	14	34	−16	3·6	0·25	6·9	—	—
Agar 5 %	22	34	−22	3·3	0·41	12·3	—	—
Salt 5 %	27	34	−25	3·8	0·53	13·9	7·8	6·3
Vitamins A, D, B_1								
(2) (*a*) Commercial	20	39	−16	3·5	0·26	7·5	—	—
sucrose 90 %	28	37	−29	3·4	0·38	11·1	—	—
Agar 5 %	35	38	−35	3·4	0·51	15·0	—	—
Salt 5 %								
Vitamins A, D, B_1								
(*b*) Same diet as (*a*)	20	33	−24	2·9	0·15	5·2	—	—
without B_1	28	31	−30	2·5	0·12	4·5	—	—
	35	32	−40	2·2	0·10	4·6	—	—
(*c*) Same diet as (*a*)	20	31	−20	3·4	0·25	7·3	—	—
paired feeding	28	32	−27	3·0	0·27	9·1	—	—
with (*b*)	35	32	−32	2·6	0·28	10·8	—	—
(*d*) Same diet as (*a*)	20	37	−21	3·5	0·15	4·3	—	—
with 59 mg. of	28	41	−24	3·4	0·13	3·8	—	—
choline daily	35	36	−30	2·9	0·13	4·4	—	—

It was, however, not as great as in the first series. In the fourth group the animals were allowed to eat as much of the diet as they wished, but choline was provided. The increase in liver fat was much less than in the first group.

DISCUSSION

The results of many experiments on large groups of animals indicate that accumulation of fat in the liver during starvation is an extremely variable phenomenon in the rat. Our stock male rats, weighing approximately 200 g., usually do not exhibit an increase in liver fat during a 48 hr. fast. On the other hand, after 1–5 days' fasting, older female rats weighing from 270 to 320 g. with large stores of depot fat may show an increase in the percentage liver fat and also in the total fat. Efforts to demonstrate an effect of choline in the latter animals met with little success. There is a suggestion of a positive result in Table I (2). In the mouse, guinea-pig, or rabbit, however, in which fatty livers are much more consistently observed after 24 and 48 hr. of fasting a definite effect of choline cannot be elicited. When animals in which fatty livers have been produced previously are fasted, choline may accelerate the disappearance of fat from the liver (Table II (1)). It is, therefore, only under these very special conditions that an effect of choline during starvation can be demonstrated. It will be understood that we are referring here to the effects of amounts of choline which may be present in a normal diet.

The results of the experiments in which the caloric intake of normal animals ingesting the diet low in choline was varied indicate, first, that with an adequate caloric intake large amounts of fat accumulate in the liver. On this adequate diet the rats consumed from 57 to 64 cal. daily per 200 g. rat. This uniformly produced a gain in weight. Secondly, if the intake is reduced to 34 cal. per 200 g. rat or to lower values, much less fat is present in the liver. Under the latter conditions the rats lose weight and there is much less depot fat present (see preliminary periods Tables II and III). When experiments are conducted on animals in which fatty livers have been produced by a low-choline diet providing an adequate caloric intake, it is seen that, with an intake in the experimental period of 37 cal. or more per 200 g. rat provided by the same diet, the liver fat continues to increase for 40 days (Fig. 1, Table II (2)); with an intake of 30 cal. or less per 200 g. rat a *steady* rise is not produced (Figs. 2 and 3, Table II (4), (5)).

The picture is quite different when a restricted caloric intake is provided during the preliminary period, and this is reduced further in the experimental period. In this case there is a much greater tendency for the liver fat to increase during the experimental period than it does when an adequate intake is given during the preliminary period and the same low intake during the experimental period (Table II (3), (5), (6), and Fig. 3). In other words, if one group is given a low intake and a second group an adequate intake of the same diet and both groups are then placed on the same restricted intake (per 200 g. rat), the liver fat of the first group shows a rise in 7 days while that of the second falls. If animals which have been on either an adequate or a restricted caloric intake of the diet low in choline during the preliminary period are placed on a very low intake of the same diet, i.e. approximately 20 cal. per 200 g. rat, the preliminary rise in liver fat during the experimental period is not observed (Fig. 2). The fall in liver fat is much more precipitous in the animals which received the adequate diet during the preliminary period. It is to be noted here also that the large reserves of body fat in animals on the adequate diet in the preliminary period (Table II (5)) did not maintain the level of liver fat any better than did the much more limited supplies in the other group of animals (Table II (6)).

In all these experiments in which the rats have received adequate or inadequate amounts of the low-choline diet for 21 days, and have been observed for a subsequent period, an important factor affecting the level of liver fat during the first part of this experimental period appears to be the *rate of weight loss*. The rate of utilization of depot fat is, of course, one of the principal factors in the loss of weight. In Tables V and VI an attempt is made to show the dependence of liver fat on rate of loss of weight. It will be observed that in both tables when the rate of loss is greater than approximately 4 p.c. of the weight at the beginning of the period, the liver fat falls. If the loss is less than this, the liver fat rises. This is true in eighteen of the nineteen observations recorded in the tables. This change in liver fat also appears to be dependent, in part at least, on the caloric intake of the animals in the preliminary period. For example, in Fig. 3 (Table II (5) and (6)) the rate of weight loss in the two groups receiving 30 cal. daily per 200 g. rat was essentially the same in 7 days, but those which previously had an adequate caloric intake lost liver fat while those which had a lower intake gained liver fat. The initial fall in liver fat when animals with an adequate caloric intake of the diet low in choline are transferred to the restricted diet and the initial rise when animals with a lower caloric intake are placed on the same restricted diet are very definite changes. No explanation of the subsequent rise of the liver fat in the animals which had previously received the adequate diet can be given except to suggest that in some unexplained

TABLE V

Experiment	Time days	Change in weight in 3 days %	Liver fat per 100 g. rat (calc.) g.	Total body fat g.	Rise or fall in liver fat in 3 days
	0*		0·64	26·5	
	3	−6	0·38	20·1	−
	0*		0·32	23·0	
	3	−2	0·40	22·4	+
	0*		1·03	17·7	
	3	−3	1·44	16·0	+
	0*		1·52	21·3	
	3	−4	1·45	—	−
	3	−4	1·72	—	+
Table III (1)	0		0·68	19·1	
	3	−5	0·57	16·1	−
(3)	0		0·72	16·8	
	3	−3	0·60	18·8	−
(4)	0		0·29	13·6	
	3	no loss	0·49	—	+
(5)	0		0·47	15·8	
	3	−2	0·68	—	+
	3	−1	0·55	—	+

* Reference is made to these experiments in the text but the figures are not given in Table III.

TABLE VI

Experiment	Time days	Change in weight %	Liver fat per 100 g. rat (calc.) g.	Total body fat g.	Rise or fall in liver fat
Table II (2)	0		0·75	26·2	
	7	−13	0·43	—	−
	7	−9	0·51	—	−
	7	−2	0·80	—	+
(3)	0		0·29	13·6	
	7	−2	0·61	13·3	+
(4)	0		0·82	18·8	
	8	−7	0·39	—	−
(5)	0		0·87	24·4	
	7	−8	0·41	—	−
	7	−4	0·61	—	−
(6)	0		0·49	11·5	
	7	−9	0·41	—	−
	7	−4	0·80	—	+

way they become "accustomed to" the restricted caloric intake. It may be pointed out, however, that the terminal fall in liver fat (Fig. 2, Table II (6)) is due to exhaustion of the reserves of depot fat. It may be emphasized here that a moderate loss of weight does not prevent deposition of fat in the liver of normal rats, i.e. those in which fatty livers have not been produced previously, when the former are placed on the diet low in choline or on the sucrose plus vitamin B_1 diet.

One of the reasons why we became interested in the effect of undernutrition on liver fat was our failure consistently to secure the results reported by **Best & Huntsman** on the rise of liver fat when animals with fatty livers were placed on a diet consisting entirely of sucrose. Since in addition to the two published experiments these investigators had observed the phenomenon in two large series of animals, it was obvious that there was a basis of fact. **Best & Huntsman** considered the possibility that the sucrose may have contained some toxic factor, but this did not appear probable since choline exerted a dramatic effect on the liver fat of the sucrose-fed animals. Choline does not prevent the acute fatty liver produced by toxic agents. Therefore instead of assuming that the sucrose we used later was free from a hypothetical toxic agent a search for a better explanation was made. After many series of experiments one probable explanation of the irregularity of results was secured. In three series of experiments in which animals on a restricted amount of the low-choline diet were transferred to sucrose, the initial rise in liver fat was observed. If the animals had been receiving an adequate caloric intake during the preliminary period, the initial rise in liver fat may or may not be observed when they are transferred to the sucrose diet. In one experiment of this type (Table III (6)) a marked rise in liver fat in the experimental period was noted which persisted for 11 days. This increase was seen in the group to which sucrose alone was given and in those which also received vitamin B_1. We have already referred to experiments of this type in which the initial rise was not observed (p. 55).

We have found, as **Best & Huntsman** reported, that normal rats placed on a diet of sucrose deposit some excess fat in their livers. The rate of deposition is much more rapid if the animals also receive vitamin B_1. In this type of experiment and also when sucrose alone was given to rats in which fatty livers had been produced previously we have confirmed the finding of our colleague, Dr **E. W. McHenry**, that vitamin B_1 favours the deposition of fat in the liver.

There are many observations recorded in this paper which perhaps merit a much more detailed discussion than we have seen fit to attempt at this time. The results illustrated in Fig. 3, for example, when two groups of rats with the same caloric intake per gram of body weight exhibit such different initial changes in liver fat, demand much further study. Determinations of metabolic rates at frequent intervals during experiments of this type may yield important clues.

SUMMARY AND CONCLUSIONS

1. The results of many experiments on large groups of animals indicate that accumulation of fat in the liver during fasting is an extremely variable phenomenon in the rat. Male rats, under the conditions of our experiments, usually do not exhibit an increase in the total liver fat although there may be a slight rise in the percentage. Large female rats usually exhibit some increase in the total liver fat under these conditions.

2. Choline in amounts not greatly in excess of that found in a normal diet exerts little, if any, effect on the deposition of fat in the liver during short periods of starvation. This conclusion applies to the female rat and also to the guinea-pig, mouse and rabbit. In the last three species the fatty liver of starvation is regularly observed.

3. In rats in which fatty livers have been produced previously, choline may accelerate the disappearance of fat from the liver during fasting.

4. Within certain limits the deposition of fat in the liver of normal rats is proportional to the caloric intake provided by the diet low in choline. In the present experiments 42 cal. or more per 200 g. rat daily gave maximum deposition in 21 days. When 34 cal. or less were given the accumulation of fat was reduced.

5. The addition of crystalline vitamin B_1 to diets low in choline or to a sucrose diet increases the deposition of fat in the liver of normal rats. These results confirm and extend the findings of McHenry.

6. A low caloric intake during a preliminary period favours the deposition of fat in the liver during the initial stages of a subsequent experimental period in which rats receive amounts of the diet low in choline providing from 26 to 30 cal. per 200 g. rat.

7. If the caloric intake has been restricted during the preliminary period, an initial rise in liver fat is noted in the experimental period in which a sucrose diet with or without vitamin B_1 is provided. This initial rise may or may not be seen if the animals consume a diet providing an adequate caloric intake during the experimental period. The results of "paired feeding" experiments show that the increase is maintained for longer periods if vitamin B_1 is given.

8. The rate of loss of weight appears to be a significant factor affecting the change in liver fat during the first part of the experimental period when animals with fatty livers are placed on diets low in choline, which provide various caloric intakes.

Experiments similar to some of those we have reported in this paper were carried out by **Elinor Huntsman Mawson** while she was working in the Department of Physiology. In an effort to extend her findings we have found it necessary to repeat some of her experiments. We would like to acknowledge our indebtedness to her.

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THE ELECTRICAL ACTIVITY OF THE CEREBELLUM AND ITS FUNCTIONAL SIGNIFICANCE

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Although some information about the electrical activity of the cerebellum was reported by Foerster & Altenburger [1935] and Spiegel [1937], the only systematic study of the problem, using modern oscillographic technique, is that briefly reported by Adrian in 1935. Apart from the paper of Beck & Bickeles [1912], in which was reported the presence of slow negative potential changes on the cerebellum following peripheral stimulation as detected by a string galvanometer, no experimental attempt has been made to correlate this activity with any aspect of cerebellar function. Our purpose has been to answer the three following questions:

(1) What are the characteristics of the electrical activity of the cerebellum?

(2) How may this activity be modified?

(3) Has this electrical activity any relationship to motor function?

Material and methods

Fourty-four cats were used in this study. Decerebration with an exposure of the anterior lobe of the cerebellum was the usual preparation. In some instances the "encéphale isolé" preparation of Bremer [1937] was used, and in others simple ether anaesthesia was employed. In some cases the brain stem was sectioned at the level of the superior colliculus but only one cerebral hemisphere was removed; this allowed the opening of the carotid arteries. The cerebellar hemispheres were exposed either by an extension of the anterior lobe exposure or by a primary removal of the bone overlying the posterior surface of the cerebellum. The cerebellar cortical potentials were led off by a pair of cotton wicks soaked in Ringer's solution usually placed 4 mm. apart on the exposed surface of the cerebellum. The potentials from the cerebellar nuclei were led off by a pair of silver electrodes insulated except at their tips and passed into the nuclei. After amplification, the potentials were recorded by a Dubois oscillograph. A five-stage condenser-coupled amplifier was generally used. The coupling capacities were small ($0{\cdot}1\,\mu$F.), in order to eliminate irregular very slow fluctuations of potential, which were found to be of no interest for the present study. The leads usually

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[1] Fellow of Commission for Relief in Belgium Educational Foundation.

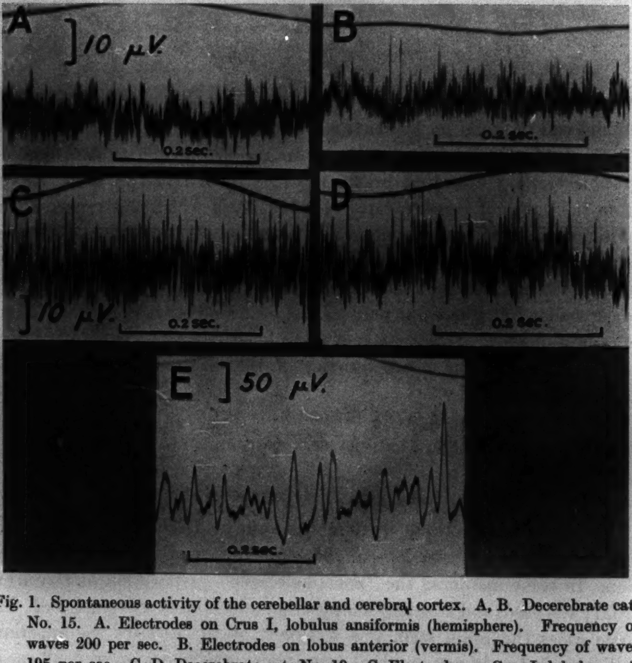

Fig. 1. Spontaneous activity of the cerebellar and cerebral cortex. A, B. Decerebrate cat, No. 15. A. Electrodes on Crus I, lobulus ansiformis (hemisphere). Frequency of waves 200 per sec. B. Electrodes on lobus anterior (vermis). Frequency of waves 195 per sec. C, D. Decerebrate cat, No. 19. C. Electrodes on Crus I, lobulus ansiformis (hemisphere). Frequency of waves 165 per sec. D. Electrodes on lobus anterior (vermis). Frequency of waves 170 per sec. E. "Encéphale isolé" cat, No. 41. Electrodes on the ecto-sylvian gyrus of the cerebral cortex (acoustic area). Frequency of waves 30–35 per sec.

served for stimulation after shunting the amplifier. A Boulitte inductorium with the primary in series with a 2 V. battery was used for stimulation. The frequency of shocks was about 40 per sec., and the strength slightly greater than the usual threshold for cerebral cortical stimulation. The electrodes were firmly fixed on the skull by means of sealing-wax so that slight movements of the head did not produce any artefact.

EXPERIMENTAL RESULTS

Spontaneous activity

As Adrian has described, cerebellar action potentials occur in groups of waves at a frequency of 150–250 per sec. The voltage of these waves was extremely variable from animal to animal in our experiments, being usually about 10 μV. For recording cerebellar action potentials the sensitivity of the amplifiers was increased to about six times that ordinarily employed for recording action potentials from the cerebral

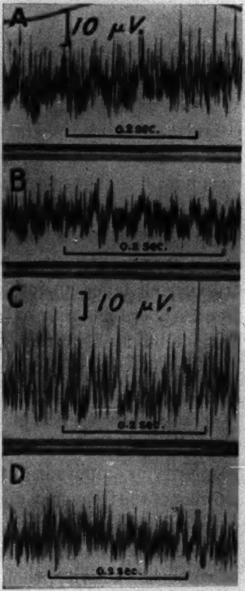

Fig. 2. Action potentials of the cerebellar cortex and nuclei. A, B. Decerebrate cat, No. 19. A. Cotton-wool electrodes 11 mm. apart, one on the lobus anterior and the other on Crus I, lobulus ansiformis. Frequency of waves 185 per sec. B. Cotton-wool electrodes 1 mm. apart on the same folia of the lobus anterior. Frequency of waves 165–210 per sec. C, D. Decerebrate cat, No. 32. C. Silver electrodes insulated except at their tips and applied to the surface of the lobus anterior. Frequency of waves 155 per sec. D. Same electrodes immediately after being placed in the interior of the cerebellum. The tip of one was located in the lateral part of the right nucleus fastigius, the other in the medial part of the left nucleus interpositus, as verified by gross examination after fixation of the brain in formalin. Frequency of waves 210 per sec.

cortex. The contrast between this activity and that of the cerebral cortex, especially in frequency of waves, is obvious (Figs. 1, 5 A and 7 E) and has been emphasized by **Adrian** [1935].

The activity of the anterior lobe (spino-cerebellar area) and the lobulus ansiformis (ponto-cerebellar area) was identical in the same experiment (Fig. 1 A, B, C, D). Reducing the distance between the electrodes from 11 to 1 mm. caused only a reduction in the amplitude of the waves but no change in their frequency (Fig. 2 A, B). This may be explained, at least in part, by a decrease in resistance between the electrodes. In the two experiments in which the comparison was made, the potentials from the depths of the cerebellum (nucleus interpositus) showed a lower amplitude and even greater frequency than those recorded from the surface of the cerebellum (Fig. 2 C, D).

Modification of activity

Cerebellar activity is very sensitive to changes in arterial pressure. When pressure is low anything which raises it, such as ephedrine or stimulation of a peripheral nerve, will cause an increase in the amplitude of the activity (Fig. 3 D, E). Peripheral stimulation of many types was used but no change in cerebellar activity was seen so long as there was no coincident change in arterial pressure. Electrical stimulation of peripheral nerves, vigorous crossed extensor reflexes in both the anterior and posterior extremities, or stretching of a large isolated muscle (vasto-crureus) all failed to induce any detectable change in the amplitude, frequency or regularity of the action potentials of either the anterior lobe or the lobulus ansiformis.

A corollary to the absence of visible effect from peripheral stimulation was the fact that, so long as the circulation was not altered locally or generally, acute section of the brain stem both caudal and rostral to the cerebellar peduncles in six experiments failed to change the frequency, regularity or amplitude of the action potentials (Fig. 3). Indeed, a slight slowing of the frequency and an increased tendency to periodicity was the only change seen following acute incomplete section of the cerebellar peduncles in four experiments. **Spiegel** [1937] noted the continuation of cerebellar activity, though at decreased amplitude, after peduncular section, but no control of blood pressure was reported.

Cocaine (2 % solution) applied to the cerebellum lowered the amplitude and often the frequency of the action potentials (Fig. 4 A, B, C). Strychnine (1 %) had a similar though less marked action (Fig. 4 D, E, F). Physostigmine, in one experiment in which three tiny crystals of the drug were placed on the anterior lobe at the site of derivation, had a similar action on the electrical activity. Although it is an unsatisfactory method and allows no conclusions, the action potentials of the cerebellum were not changed when 1/100,000 acetylcholine was dropped on the surface of the anterior lobe at the site of derivation.

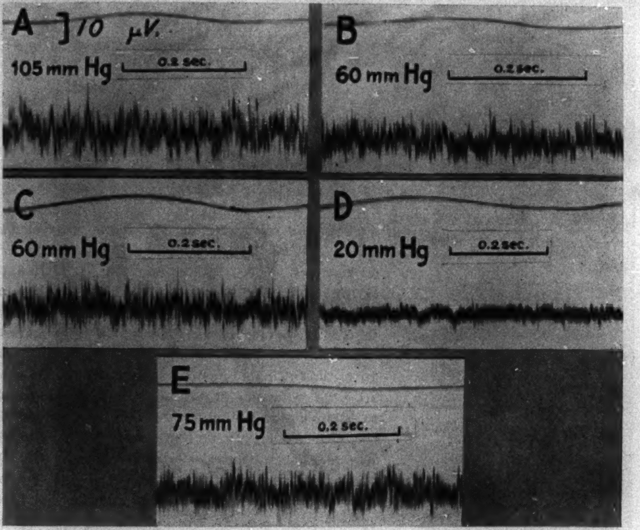

Fig. 3. The effect of blood pressure on cerebellar activity and the negative effects of isolation of the cerebellum from caudal and rostral levels. Decerebrate cat, No. 13. Derivation by cotton-wool electrodes from the lobus anterior. A. 10.45 a.m. Before ether given. B.P. 105 mm. Hg. B. 10.50 a.m. During ether anaesthesia to abolition of the corneal reflexes and almost to respiratory arrest. B.P. 60 mm. Hg. C. 10.55 a.m. Immediately after section of the medulla just caudal to the inferior cerebellar peduncle. B.P. 60 mm. Hg. D. 11.00 a.m. After the circulatory collapse induced by the medullary section. B.P. 20 mm. Hg. 3 mg. ephedrine given intravenously at 11.01 a.m. E. 11.12 a.m. Return of cerebellar activity with the return of the blood pressure. B.P. 75 mm. Hg. Detailed examination of the whole record showed that there was no consistent change in the activity in amplitude, frequency or regularity except as could be explained by the fall in blood pressure.

In the decerebrate or "encéphale isolé" preparation the administration of a general anaesthetic caused such a fall in blood pressure that it was impossible to judge whether the decrease in activity was due directly to the anaesthetics or indirectly to the fall in blood pressure. A greater concentration of ether vapour applied locally by bridging over the site of derivation with an ether soaked cotton pad, a procedure suggested by Prof. Bremer, caused a marked depression in activity separate from any fall in blood pressure. In an animal without decerebration or spinal section, simultaneous recording of the activity of the cerebral cortex (ecto-sylvian gyrus) and the cerebellum (uvula and lobulus

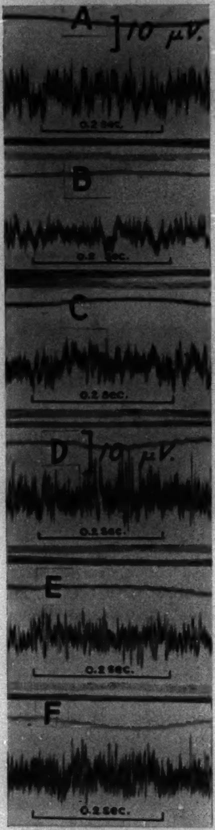

Fig. 4. The effect of cocaine and strychnine on cerebellar activity. A, B, C. Decerebrate cat, No. 23. Electrodes on the anterior lobe. B.P. 90 mm. Hg. A. 11.15 a.m. Before application of cocaine. B. 11.40 a.m. 10 min. after the application of 2 % cocaine to the anterior lobe for 5 min. This record was taken at the most marked depression of activity. The decerebrate rigidity was visibly and palpably increased. C. 12.06 p.m. 36 min. after the application of cocaine. The rigidity returned to its previous state along with the return of activity in the anterior lobe. D, E, F. Decerebrate cat, No. 15. Electrodes on the anterior lobe. B.P. 45 mm. Hg. D. 12.09 p.m. Before strychnine. E. 12.31 p.m. 3 min. after 1: 100 strychnine sulphate solution had been applied to the anterior lobe for 1 min. F. 12.42 p.m. Return of normal activity but no appearance of "strychnine spikes" in either records E or F.

paramedianus) showed that the activity of the cerebral cortex was much more affected by general ether anaesthesia than was the cerebellar activity (Fig. 5 A, B). In some cases there was an increase in the amplitude of the

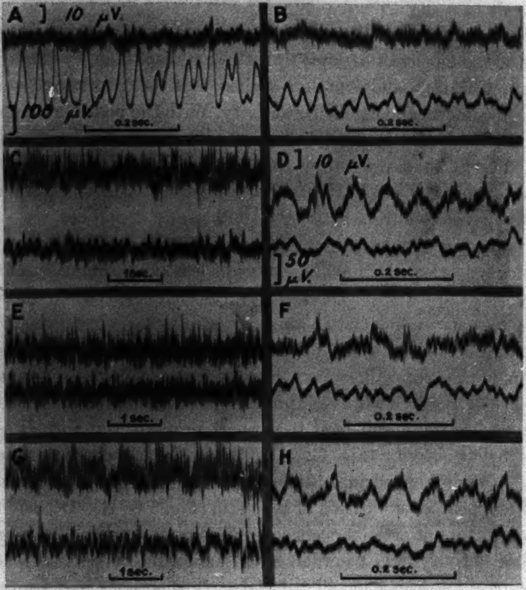

Fig. 5. A, B. The effect of ether anaesthesia on the cerebral and cerebellar action potentials. Intact cat, No. 41. Cerebellar record above, electrodes on the uvula and lobulus paramedianus. Cerebral record below, electrodes on the ecto-sylvian gyrus. B.P. 80 mm. Hg. A. Light ether anaesthesia with spontaneous movements present. B. Deep ether anaesthesia with respiratory arrest but without a fall in blood pressure. C.–H. Slow waves in the cerebellar activity under poor circulatory conditions. "Encéphale isolé" cat, No. 44. Cerebellar record above, electrodes on the uvula and lobulus paramedianus. Cerebral record below, electrodes on the ecto-sylvian gyrus. C, D. Records at slow and fast speed to show the "abnormal" cerebellar activity. B.P. 50 mm. Hg. Note the poor condition of the cerebral cortical activity as compared to A. E, F. Same preparation 10 min. after 2 mg. ephedrine given intravenously. Note the improvement of the cerebral activity and the disappearance of the slow rhythm from the cerebellar activity. B.P. 90 mm. Hg. G, H. Same preparation after repeated asphyxia had permanently depressed the blood pressure and damaged the general condition of the preparation. B.P. 70 mm. Hg. Note the reappearance of the slow cerebellar rhythm.

cerebellar waves; this was not considered to be a true augmentation of activity, but simply a better synchronization induced by the anaesthetic agent. Adrian [1935] has described a greater regularity of the waves following chloroform anaesthesia. Such a tendency was seen in ether anaesthesia to a slight degree (Fig. 5 A, B).

The "encéphale isolé" preparation proved very useful for the comparison of the effects of arterial hypotension, asphyxia by the arrest of artificial respiration, and complete occlusion of the carotid and vertebral arteries on cerebral and cerebellar activity. With the possible exception of a continued hypotension, cerebellar activity tended to be slightly less disturbed by all these procedures than cerebral activity. Both, however, were completely abolished at a stage of asphyxia in which the animal was in a convulsive state from activity of bulbar and spinal centres. Any of these damaging influences may induce in the cerebellar activity a "pathological" slow rhythm. This more or less synchronous discharge at frequencies of 15–30 per sec. was never seen except when there were obvious reasons for poor circulatory or respiratory conditions. It was repeatedly induced by occlusion of the carotid and vertebral arteries or by stopping the artificial respiration in the "encéphale isolé" preparation (Fig. 7 F, G). Such slow waves appeared in one experiment in which the blood pressure was insufficient to support properly the normal cerebral and cerebellar activity (Fig. 5 C, D). When the blood pressure was improved by giving ephedrine the slow rhythm disappeared (Fig. 5 E, F), only to reappear after a period of asphyxia (Fig. 5 G, H). Occasionally in the experiments of arterial occlusion or asphyxia, in which cerebral and cerebellar activity was recorded simultaneously, activity in the cerebellar cortex was seen when all cerebral cortical activity had disappeared. Sometimes, indeed, by virtue of the appearance of the slow waves, the amplitude might even be greater than before asphyxia (Fig. 7 F, G). This is not considered to be a stimulating effect of asphyxia or a true augmentation of cerebellar activity, but simply a "pathological" tendency to synchronous discharge at low frequencies. This interpretation is suggested by the recent observation of Adrian [1937] on the optic ganglion of *Dytiscus*.

In other experiments, usually with decerebrate preparations, after several hours of exposure and repeated faradization, there appeared spontaneous irregular waves of great amplitude (Fig. 6 B). They were never seen early in an experiment and were again thought to be an expression of a pathological tendency for synchronous discharge. This may be the explanation of the slow waves in the cerebellum described by

Foerster & Altenburger [1935] and by Spiegel [1937]. The latter reported that the slow waves often appeared only late in an experiment.

Strychnine applied to the cerebellum in strength of 1/1000 failed to modify activity in eight experiments. In 1/100 solutions it regularly caused a depression of activity as mentioned above (Fig. 4 D, E, F). The huge waves of synchronous discharge seen in the cerebral cortex action potentials after application of strychnine, the so-called "strychnine spikes" described by Gozzanno [1935], Bremer [1935*b*] and Dusser de Barenne & McCulloch [1938], were never seen in the cerebellar activity. In one experiment, however, after the spontaneous waves similar to those shown in Fig. 6 B had appeared, the application of 1/1000 strychnine did cause a change in the activity. This consisted of the appearance of groups of large rhythmic waves at a frequency of about 70 per sec., but even here nothing resembling the so-called "strychnine spikes" was seen (Fig. 6 C, D).

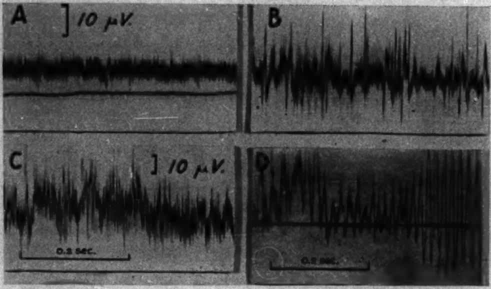

Fig. 6. A, B. Spontaneous activity early and late in an experiment. Decerebrate cat, No. 36. Electrodes on the anterior lobe of the cerebellum. Time in seconds. A. 11.30 a.m. 1½ hr. after exposure and before any local faradization. B. 3.30 p.m. 5½ hr. after exposure and after repeated local faradization. When the large irregular waves seen in B are seen in fast speed they resemble in their individual form those seen in the cerebral cortex. C, D. The effect of strychnine sulphate 1 : 1000 on a preparation already showing "abnormal" activity as in B. Decerebrate cat, No. 28. Electrodes on the anterior lobe. C. 10.30 a.m. 30 min. after exposure of the cerebellum. Repeated attempts to modify this kind of activity in other experiments by 1 : 1000 strychnine were unsuccessful. D. 5.30 p.m. 7 hr. later. Spontaneous abnormal waves had been present such as seen in B for several hours. Record D taken immediately after application of 1 : 1000 strychnine.

Faradic stimulation of the cerebellum at the site of derivation never induced the low-frequency waves seen following such a procedure on the cerebral cortex. Here, as Adrian has shown, strong stimulation was followed by an activity of increased amplitude, and greater regularity and usually increased frequency (Fig. 7 A, B, C, D) (in one instance in this series 300 per sec.). This was in turn followed by a period of decreased activity which lasted for 1 or 2 min. before it returned to its previous level of activity. If the stimulus were shorter or weaker, instead of the increase in activity, there was apparently a primary depression of activity with decreased amplitude and often decreased frequency. It is

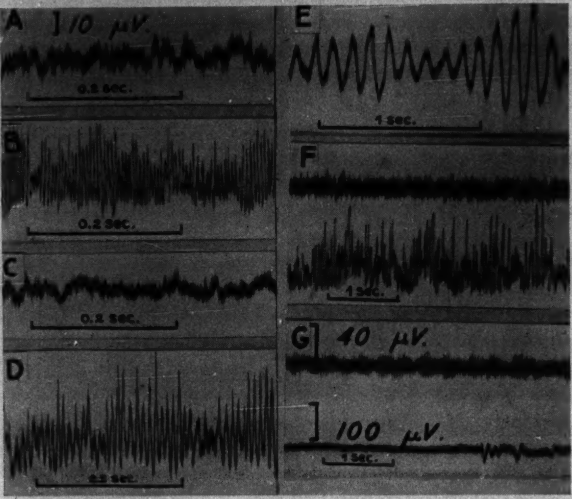

Fig. 7. A–D. Post-stimulatory augmentation of activity of the cerebellar cortex following continued faradic stimulation early and late in an experiment. Decerebrate cat, No. 36. Electrodes on the anterior lobe. A. Before stimulation. B. 4 sec. after a stimulus applied at the site of derivation of 10 sec. duration. Secondary coil at 22½ cm. C. Same experiment 4 hr. later. Before stimulation. D. 5 sec. after an identical stimulus to that given before B. Note the marked reduction in the frequency of the waves and the tendency to periodicity. E. Example of activity of the cerebral cortex. Note that here time is in seconds. F, G. Effect of occlusion of the carotid and vertebral arteries for 1 min. on cerebellar and cerebral activity. "Encéphale isolé" cat, No. 43. Cerebellar record above, electrodes on the uvula and lobulus paramedianus. Cerebral record below, electrodes on the ecto-sylvian gyrus. F. Before occlusion of the blood supply for 1 min. G. 15 sec. after the release of the carotid and vertebral arteries. Note the appearance of low frequency waves in the cerebellum at a time when the cerebral activity was still completely abolished.

necessary to state that in both cases, because of the necessity of shunting the amplifier during the stimulation, there was a delay of 2–3 sec. immediately after the stimulus to allow the amplifiers to become stabilized, and during this interval we have no information as to the state of cerebellar activity. It should be emphasized that this augmentation of activity was quite different from the increased amplitude of the discharge at low frequency brought on by asphyxia. To get the augmentation effect following stimulation, the condition of the preparation must be excellent. Thus, although synchronization appeared to be induced, it was at a very high frequency similar to that present during spontaneous activity under favourable conditions (Fig. 7 A, B). We have noted in a few experiments that after several hours the rate of this post-stimulatory discharge may diminish (Fig. 7 C, D) but never to the low frequencies seen during asphyxia. Periodicity may also occur (Fig. 7 D, G) resembling slightly cerebral activity (Fig. 7 E), but the frequency of the waves is very much greater in the cerebellum.

Adrian stated that this augmentation effect spread "some distance" over the cerebellar cortex. We have found that a stimulus of sufficient strength to cause a marked effect when applied at the site of derivation was without any effect when applied 4 mm. from the site of derivation. This is in contradistinction to the cerebral cortex and may be explained by the absence in the cerebellum of long association tracts [Dow, 1936].

Electrical activity and cerebellar function

The most widely studied function of the cerebellum is the influence of the anterior lobe on postural tone. It has been well established that stimulation of it causes a diminution in the normal or exaggerated tone of the extensor muscles in the thalamic or decerebrate cat, and that extirpation of the anterior lobe or its anaemic destruction causes the extensor rigidity to be markedly increased [for literature see Bremer, 1935*a*]. Magoun *et al.* [1937] have shown that cocaine injected into the anterior lobe also causes an increase in the rigidity. Bremer in 1922 described in detail the characteristics of the rebound following a stimulation of the anterior lobe. He showed that if the stimulus was short the post-inhibitory rebound was rapid and the tonus was maintained at a higher level than before the cerebellar stimulation. If the stimulus was longer or repeated sufficiently in quick succession, the rebound became dissociated into two components, one a quick, jerk-like contraction followed by relaxation and then a slow progressive augmentation of tone up to its pre-stimulatory level, or higher. If the stimulus was further strengthened or prolonged, all trace of the quick rebound would disappear and after an interval of several seconds the tonus would gradually rise to its former level or above. We have confirmed all these observations.

In the present series, simultaneous recording of the post-stimulatory electrical activity of the anterior lobe and the tension in an extensor muscle (plantaris) in a decerebrate preparation has shown that, other things being equal, the two are inversely proportional. A stimulus so short as to cause only a quick and sustained rebound was followed by a primary phase of depressed activity. Coincident with the return of the activity of the anterior lobe to its pre-stimulatory state the muscle tonus gradually fell. Then so long as the cerebellar activity was stable the tonus remained the same. Fig. 8 A–F illustrates such an experiment. Fig. 8 A shows the level of tonus in the plantaris, recorded by an optical system on an isometric myograph, and the pre-stimulatory cerebellar activity. In B four successive interrupted stimuli were given (only two shown), the amplifiers meanwhile being shunted. Each stimulus was followed by a rebound and a plateau of increased tonus. At the end of the fourth stimulus the activity of the anterior lobe was recorded three seconds after the end of the last stimulus. A comparison of the records of muscle and cerebellar activity here with that in A shows that the muscle tonus was increased and the cerebellar activity was decreased. C, D and E are successive pieces of a continuous record. Although the interval between D and E is shortest, the fall of tonus here was greatest. This is the point at which the cerebellar activity returned to the level seen in A. F was taken 37 sec. later and shows that with the stabilization of the cerebellar activity the tonus was also stabilized.

The same correlation between functional activity, as measured by the muscle tone, and electrical activity of the inhibitory area of the anterior lobe was demonstrated by the application of cocaine to the anterior lobe. As stated above, the application of cocaine (2 %) to the anterior lobe depressed the electrical activity (Fig. 4 A, B, C). This was associated with an increase in the decerebrate rigidity as measured by palpation of the limbs. After about 40 min. coincident with the return of electrical activity the animal returned to its previous state of rigidity.

When the stimulus given to the anterior lobe was long and continuous, there was a post-stimulatory augmentation of electrical activity and a dissociation of the rebound or its complete absence, as if the exaggerated spontaneous activity of the cerebellum which followed its stimulation had the same effect as the stimulation itself. During the phase of depression of cerebellar activity the muscle tonus rose to above normal and then fell to a stable level near its pre-stimulatory level coincident with the return of electrical activity in the anterior lobe. Fig. 8 G, H, I, J are samples of a continuous record showing the various phases of activity following a continuous stimulus (same experiment as Fig. 8 A–F). Note in H the augmentation of electrical activity and a decrease in tone so marked that the muscle record is below the recording paper. The tonus gradually rose

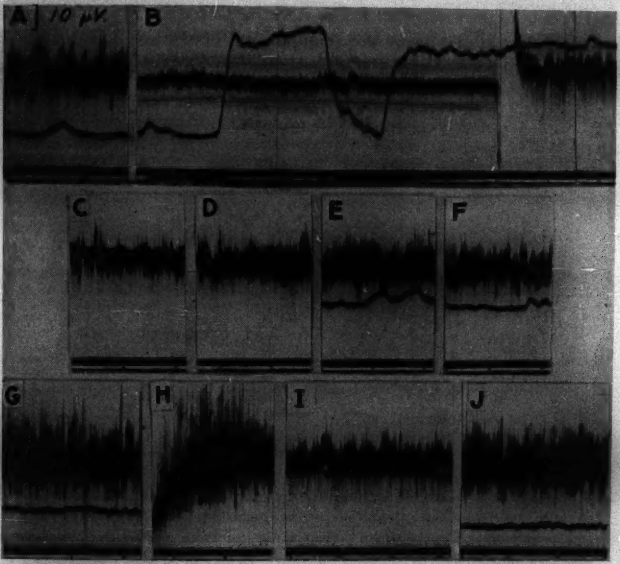

Fig. 8. Simultaneous record by an isometric myograph of an extensor muscle (plantaris) and the action potentials of the anterior lobe of the cerebellum. Decerebrate cat, No. 27. Stimulation in B at 22·5 cm. marked by signal at bottom of record. Electrodes on the anterior lobe of the cerebellum. A. Before stimulation. B. During and after four interrupted stimuli of 1 sec. each (only two shown on published record). C. 19 sec. later. D. 12 sec. later. E. 9 sec. later. F. 37 sec. later. Note that the rate of fall is greatest between D and E, which is coincident with the return of the action potentials to their pre-stimulatory state. The amplifiers were shunted during the stimulation, record B. (The myogram shows those adventitious irregularities which are apt to occur at the end of a long experiment.) G. Before stimulation. H. Augmentation phase. Record 4 sec. after end of a continuous stimulation for 20 sec. at same strength as in B. Muscle record depressed below recording paper. I. 7 sec. later. Depressed phase with increased tonus. J. 60 sec. later after stabilization of activity and tonus.

to the level seen in I, the depressed phase, and dropped again one minute later coincident with the return of cerebellar activity.

A record to show more clearly the dissociation of rebound and the correlation of the slow phases of the response with the activity of the anterior lobe is shown in Fig. 9. The stimulus lasted for 15 sec., the amplifiers being shunted during the stimulation. The tonus fell during the stimulation (9 A), showed a slight rebound at the end of the stimulus (9 B), but immediately fell again. During this phase of the post-stimulatory effect the cerebellar activity was increased to three or four times its previous amplitude. As the activity diminished the tonus gradually rose much above its pre-stimulatory level. C is a sample of activity during the depressed phase. No muscle record is seen because the tonus level was so high that its record was above the recording paper. D and E show its gradual return to a stable level coincident with the return of the cerebellar activity, and F shows the stabilization of both tonus and cere-

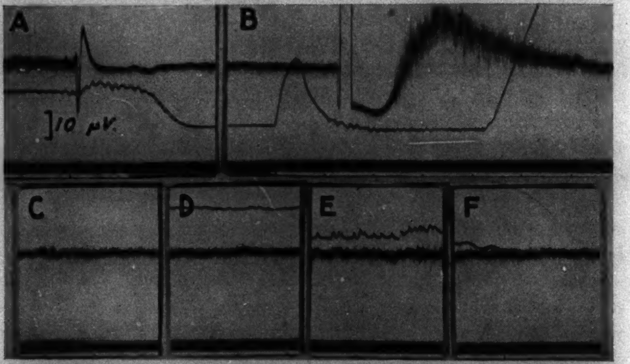

Fig. 9. Simultaneous record of an isometric myogram of the plantaris muscle and the electrical activity of the anterior lobe of the cerebellum before, during and after continuous stimulation at the site of derivation. Decerebrate cat, No. 37. Amplifiers on shunt during stimulation. Note the relaxation during stimulation followed by a brief rebound and a second relaxation to the stimulation level. The tonus then rose as the activity diminished and was above the recording paper while C was being taken. Gradually returned coincident with the return of cerebellar activity to become stabilized in F. A. Before stimulation, and at beginning of stimulation. B. 12 sec. later, showing end of stimulation. C. 15 sec. later. D. 40 sec. later. E. 40 sec. later. F. 40 sec. later. Repeated control observations have shown that the augmentation of activity seen in B is not due to any temporary increase in sensitivity.

bellar activity. Because the above records were taken at slow speed it is impossible to see the individual waves of the cerebellar oscillogram. By taking a discontinuous record at fast speed at different points in the post-stimulatory response it was possible to show that during the time of depression of tonus we have a true augmentation effect. Fig. 10 is such a record taken from the same experiment and immediately after the record shown in Fig. 9.

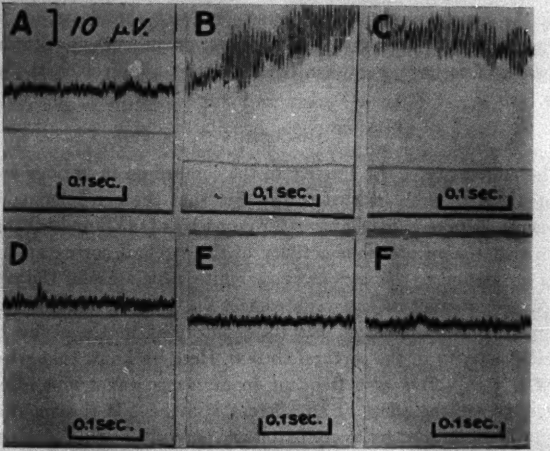

Fig. 10. Record taken of an identical experiment on the same animal and immediately after the record shown in Fig. 9. Fast speed of recording to show individual waves. A. Before stimulation. B. 2 sec. after stimulation. Muscle tone at the same level as it was during the 15 sec. continuous stimulation. C. 4 sec. after stimulation. Augmentation of activity and relaxation of extensors continues. Waves 170 per sec. D. 6 sec. after stimulation. The muscle tone is gradually increasing as the cerebellar activity diminishes. E. 30 sec. later. Muscle record is above the recording paper. F. 90 sec. later. Stabilization of activity and tonus near the pre-stimulatory level.

The association of the post-stimulatory augmentation with the absence or dissociation of rebound was shown in quite another way by alternately cooling and warming the surface of the anterior lobe in one experiment. When the anterior lobe was cooled a stimulus of 15 sec. duration was followed by a quick and sustained rebound. After the application of hot saline an identical stimulus resulted in a quick phase of the rebound followed by a depression of tonus and then a slow rise (dissociation of rebound). It was then observed, as would be expected, that in this experiment cooling of the anterior lobe prevented the augmentation effect. When the anterior lobe had been warmed augmentation reappeared after the same stimulus was applied.

In the majority of experiments the best effects were obtained when the whole anterior lobe was exposed and the electrodes for stimulation and derivation were placed one on either side. In other cases only the homolateral side gave good results. These effects were obtained both when the decerebration was made above and below the red nucleus. When the same procedure was carried out with the electrodes on the lobulus ansiformis (hemisphere), good post-stimulatory augmentation and depression of activity were seen but there was no effect on the muscle tonus.

DISCUSSION

The uniformity of the activity of the cerebellum in various parts of the organ is probably related to the fact that the cerebellar cortex is uniform in histological structure. Kornmüller [1937] and others have shown that the spontaneous activity of the cerebral cortex apparently varies in relation to the various cyto-architectonic areas of the cerebral cortex. The absence of any visible effect on the cerebellar oscillogram from peripheral stimulation has its counterpart in the study of the electrical activity of the cerebral cortex. Here we know that when the cortex is very active with frequent spontaneous waves it is often impossible to see the effect of an afferent stimulus. If the spontaneous activity is diminished by sleep, barbituric narcosis, or the local application of cocaine, responses which were previously masked appear clearly. In the cerebellum also the very rapid spontaneous activity possibly masks any effects produced by the peripheral stimulation. The positive findings of Beck & Bickeles [1912] regarding slow potential changes in the cerebellar vermis following peripheral stimulation might be explained by a change in blood pressure. But this would not explain their negative findings on the cerebellar hemisphere or the occipital lobe which we have shown are equally sensitive to blood pressure changes. The recording system used in our own experiments is so different from that of these early workers that comparison is difficult.

In the cerebellum, as Adrian [1937] has pointed out for the optic ganglion of the *Dytiscus*, and Bremer [1938] for the area striata of the cerebral cortex, synchronization and increased amplitude of waves does not necessarily mean increased functional activity. Indeed, in the cerebellum, under distinctly unfavourable conditions of circulation and respiration, we have repeatedly seen just before and just after the disappearance of all electrical activity, an increased synchronization and, probably as a result, an increased amplitude of the potentials, but under these circumstances the frequency was always reduced. We are convinced that increased amplitude of action potentials means increased functional activity only when the frequency of the waves remains unreduced or is increased. One must admit, however, that it is possible, due to the peculiarities of synaptic physiology, that a smaller number of neurones acting synchronously can cause the discharge of a second neurone when a greater number of neurones beating asynchronously are unable to. The post-stimulatory augmentation effects here described are, by all criteria, an indication of increased function since the action potentials show increased frequency, amplitude and synchronization.

The relatively long periods of decreased cerebellar activity following stimulation may explain the equally long periods of post-stimulatory tonic "rebound" activity seen by Hare *et al.* [1937] after stimulation of the deep cerebellar nuclei in cats and monkeys, and by Clark [1937] after stimulation of the cerebellar cortex in the unanaesthetized cat. If this were the explanation the effects described by these workers would be release phenomena. Miller & Laughton [1928] have looked upon the rebound following cerebellar nuclear stimulation as a release phenomenon. Magoun *et al.* [1937], comparing the rebound observed after stimulation of the interior of the cerebellum with the release phenomenon which follows destruction of the cerebellum, were struck by certain differences. They were of opinion that cerebellar rebound was not a release phenomenon. The fact that local stimulation does not cause any subsequent change in the electrical activity of areas remote from the site of stimulation, would argue against the idea advanced by Clark that the complex orderly movements which he observed involving the whole musculature are due to a spread of the effect of stimulation from one cortical area to another. If the continued motor phenomenon described by Clark [1937] is due to this essentially local post-stimulatory depression of activity the fact that the whole musculature is involved in the phenomenon argues once more against a strict topographical localization in the sense of Mussen [1927, 1930]. The effects on decerebrate rigidity described here, following changes in the electrical activity of the anterior lobe and their absence following similar changes in the activity of the lobulus ansiformis, support the conception of functional localization in the cerebellum as given by Bremer [1935*a*] and recently outlined by Fulton & Dow [1937].

The state of activity of the anterior lobe will not explain the whole of cerebellar rebound. The quick phase of the dissociated rebound described by Bremer in 1922 is seemingly unrelated to the cerebellar activity. This contraction occurs at a time when, although due to mechanical difficulties we cannot record the activity, we have every reason to believe the cerebellar activity is at its highest post-stimulatory phase for that particular stimulus. In many experiments the actual level at which the tonus became stabilized coincidently with the stabilization of the cerebellar activity was slightly higher than the pre-stimulatory level. This we look on as a true shortening reaction and not dependent on the state of cerebellar activity at a particular moment. In an occasional experiment these two factors and possibly others all unrelated to the cerebellar activity partially obscured the relationship so clearly shown in the majority of the stimulation experiments.

The oscillographic study of the activity of the cerebellum has shown that in so far as the anterior lobe is concerned the action potentials are a direct indication of the functional state of this part of the cerebellum. The work indicates that this activity is linked with a motor function of the cerebellum, namely, the inhibitory effect of the anterior lobe on postural tone.

SUMMARY AND CONCLUSIONS

1. A detailed study of the spontaneous electrical activity of the cerebellum is reported. This activity is uniform throughout the cerebellum and consists of small ($\pm 10 \mu$V.), rapid, irregular oscillations of potential, amongst which can be seen groups of regular waves of the same high frequency (150–250 per sec.).

2. The spontaneous activity of the cerebellum is apparently not modified by any afferent stimulation provided blood pressure is not altered.

3. Except for a slight slowing of the frequency and an increased tendency to periodicity, the cerebellar oscillogram is not changed by complete section of almost all afferent fibres to the cerebellum so long as the blood supply is not damaged.

4. The cerebellar activity is extremely sensitive to local circulatory changes, asphyxia, and lowered systemic blood pressure.

5. Under adverse conditions of circulation and respiration cerebellar waves may occur at much lower frequency and occasionally at greater amplitude than during more "normal" conditions. This is considered to be a "pathological" tendency to synchronization at a slow frequency and

is differentiated from a true augmentation of activity such as is seen immediately following a strong faradization of the cerebellar cortex.

6. Faradic stimulation of the cerebellar cortex at the site of derivation is followed by a period of diminished activity, if the stimulus is relatively weak or short. If the stimulus is made longer or stronger, the primary effect is a true augmentation of activity with increased amplitude, up to 3 times the resting value, a frequency up to 300 per sec. and synchronization of the waves. This is in turn followed by a much longer period of diminished activity.

7. These post-stimulatory changes in the electrical activity of the inhibitory area of the anterior lobe of the cerebellum are reflected in the postural tone of an extensor muscle (plantaris) in decerebrate rigidity. Other factors being equal, the postural tone is inversely proportional to the degree of activity measured by the frequency and amplitude of the waves in the anterior lobe of the cerebellum. The same correlation between the functional and the electrical activity of the inhibitory area can be demonstrated by the cocainization of this area.

8. The oscillographic method applied to the anterior lobe of the cerebellum appears thus to record the functional activity of this part of the cerebellum.

It is with pleasure that the author expresses his gratitude to Prof. Frederic Bremer, in whose laboratory this work was done, for his stimulating suggestions and criticisms throughout the present study and during the preparation of this paper, and also to M. Tirabassi for technical assistance in this research.

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THE ACTION OF EPHEDRINE

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THERE can now be little doubt that the active substance liberated by cholinergic nerves is acetylcholine, but the substance, or substances, liberated by adrenergic nerves have not yet been identified with certainty. The experiments described below represent part of an attempt to identify them. They originated in an unsuccessful attempt to demonstrate the liberation of substances by the vasodilator nerves in a rabbit's ear. Some of the results have been already mentioned in a general discussion of the action of ephedrine [Gaddum, 1938].

Experiments designed for the demonstration of the liberation of acetylcholine have been greatly facilitated by the use of eserine, which inhibits the hydrolysis of acetylcholine by choline esterase. If a substance could be discovered which would protect adrenaline as eserine protects acetylcholine, experiments with adrenergic nerves might be made much easier. According to Easson & Stedman [1936] the action of eserine on choline esterase is due to substrate competition. Eserine and acetylcholine are both esters which both combine with the enzyme in the same way, thus entering into competition with one another. The destruction of eserine by the enzyme is a comparatively slow process, which occupies the active patches of the enzyme for a long time leaving no room for acetylcholine. It therefore seemed likely that some substance allied to adrenaline in chemical structure might fill the place of eserine in studies on adrenergic nerves. Ephedrine appears to have an action of this kind, though it does not produce such striking effects as eserine.

The suggestion that ephedrine might act in this way came from the work of Blaschko *et al.* [1937*a*], who studied the destruction of adrenaline by tissues in the presence of cyanide. This destruction is probably due to amine oxidase [Kohn, 1937; Richter, 1937]. Ephedrine is not oxidized by the enzyme [**Blaschko** *et al.* 1937*b*], but inhibits the destruction of adrenaline, presumably by substrate competition.

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Various writers have described results which might be due to the inhibition of amine oxidase by ephedrine. **Schaumann** [1928] was struck by the fact that ephedrine had much less action on isolated tissues than might have been expected from its activity in the body. He attributed this lack of activity to the absence of adrenaline, and found that when adrenaline (10^{-7}) was added to the fluid perfusing a frog's hind legs the preparation became very sensitive to ephedrine. **Burn** [1932] obtained similar results when a dog's legs were perfused with blood.

In other experiments ephedrine has been found to increase the effect of a subsequent dose of adrenaline. A potentiation of this kind has been seen in experiments on isolated uterine muscle from rabbits and on the blood pressure of dogs and rabbits [**Schaumann**, 1928; **Launoy & Nicolle**, 1928; **Reinitz**, 1929; **Munch & Hartung**, 1929; **Koppanyi & Luckhardt**, 1931] and man [**Csépai & Doleschall**, 1928]. **Pak & Tang** [1933] found that the application of ephedrine to a rabbit's conjunctiva sensitized the pupil to the subsequent local application, or injection, of adrenaline. None of these writers suggested that the potentiation was due to inhibition of an enzyme.

On the other hand, ephedrine has been found to antagonize the action of adrenaline on the perfused hindlegs of a frog [**Schaumann**, 1928], dog's blood pressure [**Curtis**, 1929], rabbit's intestine [**Finkleman**, 1930; **Thienes** *et al.* 1934; **Reinitz**, 1929], toad's intestine [**Epstein**, 1931] and dog's heart-lung preparation [**Burn & Tainter**, 1931]. It may even reverse the inhibitory action of adrenaline on virgin cat's uterus [**Curtis**, 1929].

Schaumann, who observed both potentiation and antagonism on frog's vessels found that the direction of the effect depended on the concentration of the ephedrine. Low concentrations caused potentiation and high concentrations caused antagonism. **Reinitz** [1929] came independently to similar conclusions regarding the action of ephedrine on rabbit's uterus. We have made similar observations of the effects of low and high concentrations of ephedrine in experiments with frogs' hearts and rabbits' ears. The experiments described below provide new examples of the fact that ephedrine may sensitize tissues to adrenaline, and show that it may also sensitize them to the effect of stimulating adrenergic nerves. Direct evidence that ephedrine preserves the substance liberated by adrenergic nerves is also presented.

METHODS

(a) *Perfusion of rabbit's ear*

Dissection. Large rabbits (over 3 kg.) with large ears were anaesthetized with ether. The common carotid artery was dissected out. The external carotid was tied about 0·5 cm. above the carotid sinus, and all the branches central to this point were tied and divided, except the artery to the ear, which runs laterally from the carotid at about the level of the superior cervical ganglion (see Fig. 1). This artery was left undisturbed in order to avoid damage to the post-ganglionic sympathetic nerves which run near it. The cervical sympathetic was then traced up to the superior cervical ganglion, and the post-ganglionic nerves which run laterally from the lower end of the ganglion were carefully dissected out for about 0·5 cm. The great auricular vein was then freed. After this preliminary dissection, during which the blood supply to the ear was undisturbed, the arterial cannula was tied in the carotid artery and perfusion with Locke's solution from a reservoir was started.

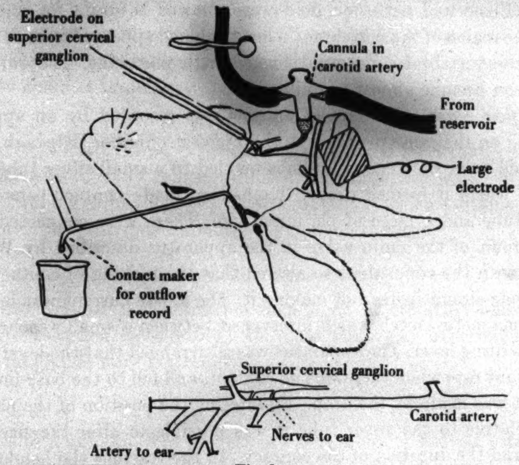
Electrode on superior cervical ganglion
Cannula in carotid artery
From reservoir
Large electrode
Contact maker for outflow record
Superior cervical ganglion
Carotid artery
Nerves to ear
Artery to ear

Fig. 1.

Arterial cannula. Fig. 1 shows the arterial cannula, which has two arms for perfusing different fluids. The cannula is largely filled with air, which separates the fluid coming from the reservoir from the fluid in the mouth of the cannula. Injections are made through the rubber cap. This type of cannula has two advantages. First, injected fluid cannot mix with fluid coming from the reservoir, and secondly, it is easy to control the rate of injection so as to avoid pressure changes by watching the fluid in the mouth of the cannula. If the injection is made too rapidly the level of this fluid rises. Control injections with the perfusion fluid itself did not affect the outflow record in any way.

Venous outflow. As soon as perfusion started a piece of thin glass tubing was tied in the great auricular vein as a venous cannula. A strong ligature was then tied round the neck and the head was removed from the body. Blood and perfusion fluid were allowed to drain for some time from the region of the vertebrae. When the blood appeared to be all washed away the vertebral canal was blocked with plasticine. The outflowing fluid soon became almost or quite clear.

Outflow record. The rate of outflow was recorded by an apparatus working on the same principle as the Pulszeitschreiber [Fleisch, 1927]. The fluid from the venous cannula was led to a small silver tube at the end of which it formed drops. Each drop made contact between the silver tube and a piece of platinum wire. These were connected in the grid circuit of the radio valve in the apparatus described by Winton [1936] with the connexions so altered that the making of contact broke the anode circuit instead of making it. The anode current was led to an electromagnetic clutch which intervened between a small synchromotor and a writing lever. The apparatus was so arranged that the lever rose at a constant rate when there was no contact, and fell to the base-line when contact was made by the drop. By altering the position of the platinum wire relative to the silver tube it was possible to alter the size of the drops and the duration of the contact. In the tracings the height of the record measures the interval between successive drops. We are indebted to Messrs C. F. Palmer who made this apparatus at our request, and who are willing to make others. The apparatus has been used regularly for many months and has given no trouble.

Stimulation of nerve. A condenser (2μF.) was alternately charged, usually to 30 V., and discharged through a resistance of 2000 Ω. by means of a rotating commutator [Hill, 1934]. The electrodes and a resistance of 10,000 Ω. were connected in series to the two ends of the 2000 Ω. resistance. One electrode was a large wet pad, and the other a platinum wire which was hooked round the post-ganglionic fibres. In this way shocks of alternating sign were applied, usually at a rate of 36 per sec., and for periods of 3–10 sec. This voltage and this rate of stimulation were found to give maximal effects.

Various methods of perfusing the rabbit's ear were tried before the method described above was adopted. The cannula was tied in the carotid artery rather than in the auricular artery itself because it was found that the flow was less easily disturbed by accidental movements of the cannula. Perfusion at a temperature of 37° did not seem to have any special advantage. Perfusion with undiluted rabbit's blood has the advantage that it makes vasomotor effects visible, but it makes the pharmacological analysis of the effluent much more difficult. Stimulation of the peripheral mixed nerves, such as the great auricular, was abandoned because the peripheral course of the adrenergic supply to the ear was found to vary, and could not always be traced. A combined perfusion of the ear and the superior cervical ganglion was attempted, but the ganglion soon ceased to conduct impulses.

(*b*) *Frog's heart*

The frog's heart (*Rana esculenta*) was suspended by Straub's method and filled with Locke's solution diluted 1·4 times with water, and containing atropine sulphate (10^{-6}). The vagus nerve, which contains adrenergic fibres was stimulated about 2–3 cm. from the heart.

(*c*) *Cat's nictitating membrane*

Contractions of the nictitating membrane of etherized and spinal cats were recorded isotonically. In these particular experiments the suprarenal glands were not removed.

(*d*) *Drugs*

Doses of adrenaline are weights of *l*-adrenaline, dissolved with HCl. Doses of ephedrine are weights of *l*-ephedrine hydrochloride.

RESULTS

When perfused by the method described above, the blood vessels of the rabbit's ear respond regularly with vasoconstriction to the injection of adrenaline or to the stimulation of the adrenergic nerves. Ephedrine, in concentrations up to 10^{-3}, has no detectable effect by itself, but when ephedrine, in a concentration of 10^{-5}, was added to the perfusion fluid, the effects of adrenaline and of stimulation of the nerves was increased.

Fig. 2 shows the increase of the response to adrenaline. Each portion of the tracing shows the effect of the injection of 0·4 μg. of adrenaline in 0·4 c.c. The third injection, which was made 6 min. after the perfusion fluid had been changed for one containing ephedrine (10^{-5}), shows an increased effect, but 10 min. later the effect was increased still further.

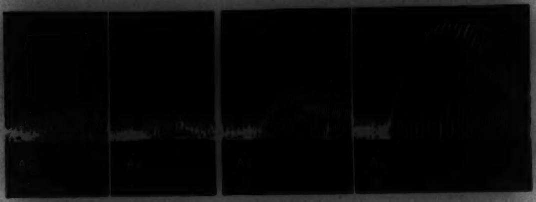

Fig. 2. Outflow from perfused rabbit's ear. The height of the record is proportional to the time interval between drops. *Ad.* injection of 0·4 c.c. adrenaline (10^{-6}). From 3.15 onwards the perfusion fluid contained ephedrine (10^{-5}).

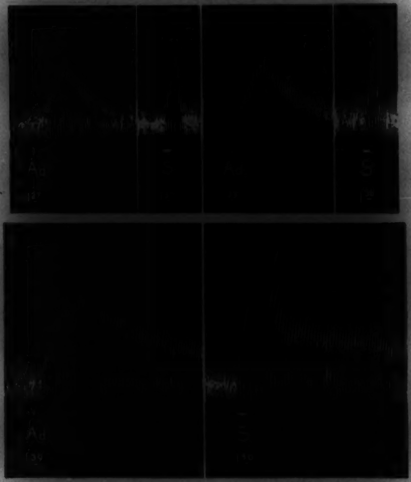

Fig. 3. Similar to Fig. 2. *Ad.* injection of 0·1 c.c. adrenaline (10^{-7}). *S*, stimulation of sympathetic, 6 sec. 25 per sec. From 1.37 onwards the perfusion fluid contained ephedrine (10^{-5}).

Fig. 3 shows the result of another experiment. The top part of the figure shows effects obtained with the normal perfusion fluid, and the bottom part of the figure shows effects obtained when the perfusion fluid contained ephedrine. *Ad.* denotes the injection of adrenaline, and *S* denotes stimulation of the sympathetic nerve. It will be seen that the vasoconstrictor response to adrenaline was increased by the ephedrine. In this particular experiment some sensitization of the response to adrenaline was seen when ephedrine had only been perfused for 2 min. In most experiments the sensitization developed more slowly than this. When the ephedrine solution was perfused for an hour or more the sensitization persisted, but it could be reversed by perfusion with ephedrine-free solution.

The same figure shows that the effect of stimulating the adrenergic nerves was also increased by the ephedrine. Stimulation of these nerves for 6 sec. at a rate of 25 maximal shocks per second caused a small and brief vasoconstriction. Fourteen minutes after the beginning of the perfusion with ephedrine this effect was increased in intensity and duration.

These effects of ephedrine appear to be a true sensitization because ephedrine itself had no vasoconstrictor action. The increase of the duration of the effects was not entirely due to the decrease in the rate of flow, which must increase the time during which adrenaline is in contact with the tissues, since in the presence of ephedrine the vasoconstriction persisted, not only for a longer time, but actually during the perfusion of a larger volume of fluid.

Fig. 4 shows the result of an experiment in which the colorimetric test for adrenaline with arsenomolybdic acid, devised in these laboratories by F. H. Shaw [1938], was applied to the fluid flowing from a perfused ear. In this experiment the rate of flow was made almost independent of vasomotor changes by the following device. The reservoir was raised to a height of 2–3 m. above the ear, and a piece of fine capillary tubing was interposed between reservoir and the ear. A simple arterial cannula was used, and no attempt was made to record the effect of injected fluids, but the effects of nervous stimulation were shown by a fine-bored water manometer connected to the cannula. With this arrangement the effect of nervous stimulation could usually still be detected on the outflow record, but the time taken to collect a sample of 10 c.c. was approximately constant at 15 min. Each sample of 10 c.c. was collected in a cylinder containing 10 c.c. of 10 % trichloracetic acid, and the apparent concentration of adrenaline was estimated and plotted as in Fig. 4. During the collection of the fourth sample the sympathetic nerves were stimulated for 5 sec. in each minute. This caused a small release of "adrenaline". After eight samples had been collected the perfusion fluid was changed for a similar fluid containing ephedrine. The ephedrine itself caused a release of "adrenaline" in these and in each of a series of such experiments. Ephedrine itself has no direct effect on the colorimetric test.

After about 50 min. this immediate effect passed off. After 114 min. the sympathetic nerves were stimulated as before and a greatly increased concentration of "adrenaline" was detected in the effluent. This substance could still be detected for more than 15 min. after the second stimulation had ceased.

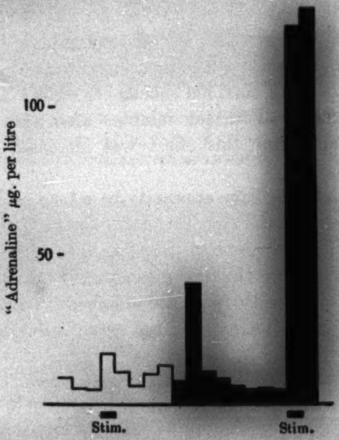

Fig. 4. Colorimetric estimates of adrenaline in successive samples of 10 c.c. of the effluent from a perfused rabbit's ear. *Stim.* stimulation of nerve for 5 sec. in each minute during collection of one sample. Black areas, perfusion with ephedrine (10^{-5}).

A special specific test was applied, in this experiment, to the third sample collected after the second stimulation. Shaw has shown that if adrenaline is subjected to suitable preliminary treatment with alkali the colour subsequently produced in the test is increased about five times. This increase appears to be a specific property of substances containing exactly the same side-chain as adrenaline, and can be made the basis of a specific test for this side-chain. When this specific test, as described by Shaw, was applied, an increase of colour similar to that due to adrenaline was observed.

The ordinary test makes it probable that the substances liberated by the nerve is a catechol derivative, since no other substances are known to give this test in low concentrations. The special specific test definitely excludes from consideration the substances whose molecules are identical with that of adrenaline except for the absence of the alcoholic hydroxyl group (epinine), or the absence (noradrenaline), or transposition of the methyl group (corbasil). Adrenalone is also excluded. The results strongly suggest that the substance is adrenaline itself.

Fig. 5. Cat. Ether. Nictitating membrane. *S*, stimulation cervical sympathetic, 10 sec. 6·5 per sec. *Ad.* 7 μg. adrenaline, intravenous. At 3.21 0·1 mg. ephedrine, intravenous.

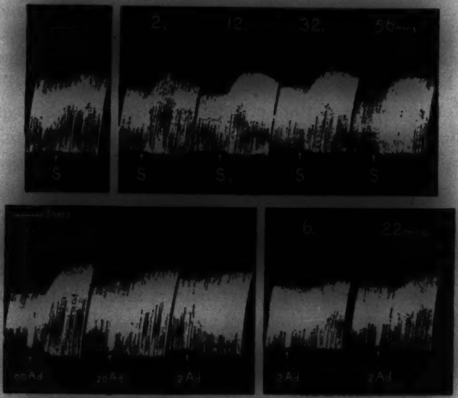

Fig. 6. Frog's heart. Straub's method. Atropine, 10^{-7}. *S*, stimulation of vagosympathetic, 4 sec. *Ad.* adrenaline. Concentrations × 10^{11}. Times after addition of ephedrine (2×10^{-9}) to fluid.

The fact that the substance detected in these experiments is not noradrenaline is particularly interesting, because of Bacq's [1934] theory that noradrenaline is identical with sympathin E, the substance liberated by excitor adrenergic nerves [Cannon & Rosenblueth, 1937]. The nerves to a rabbit's ear are excitor and might be expected to liberate sympathin E, but it would be unwise to reject Bacq's theory until we know more about the pharmacological properties of the substance liberated in the rabbit's ear.

Cat's nictitating membrane. Fig. 5 shows the effect of ephedrine on the responses of a cat's nictitating membrane. At *S* the cervical sympathetic was stimulated (10 sec.; 6·5 sec.). At *Ad.* 7 μg. of adrenaline was injected intravenously. At 3.21 0·1 mg. of ephedrine hydrochloride was injected intravenously. This caused a slow contraction of the nictitating membrane. The effects of adrenaline, and of nervous stimulation, were both increased in strength and duration. These increases developed slowly.

Frog's heart. Fig. 6 shows the effect of ephedrine on the responses of a frog's heart. The upper part of the tracing shows that the presence of ephedrine increased the effect of stimulating the adrenergic nerves, and that this increase reached a maximum in about 12 min. The lower part of the tracing shows that the effect of adrenaline was simultaneously increased and that this increase also developed slowly.

DISCUSSION

Sensitization. The evidence that ephedrine potentiates the action of adrenaline by inhibiting amine oxidase is similar to the evidence that eserine potentiates the action of acetylcholine by inhibiting choline esterase. Both drugs have been shown to protect the chemical transmitter from the enzyme *in vitro* and to potentiate its action *in vivo*, whether it is injected or liberated locally by stimulation of the nerves. Both drugs may increase the yield of chemical transmitter liberated from the tissue when the nerve is stimulated.

Experiments on the effect of the degeneration of nerves confirm the theory that some of the actions of ephedrine resemble those of eserine. If the cholinergic nerves to the pupil are cut and allowed to degenerate the pupil becomes abnormally sensitive to acetylcholine [Shen & Cannon, 1936] and insensitive to eserine [Anderson, 1905]. Eserine's failure to act on the denervated pupil is easily understood if this effect of eserine is due to the inhibition of the destruction of the acetylcholine normally liberated, since the liberation of acetylcholine presumably ceases after degeneration of the nerves.

If adrenergic nerves are cut and allowed to degenerate, the pupil and various other tissues become abnormally sensitive to adrenaline [Meltzer & Auer, 1904] (for other references see Cannon & Rosenblueth, 1936). This effect is presumably analogous to the increased sensitivity to acetylcholine which may be produced by cutting cholinergic nerves. Degeneration of adrenergic nerves affects different actions of ephedrine in different ways. It greatly diminishes its action on the blood vessels of a cat's leg [Burn, 1932] and eventually decreases its action on the pupil [for references see Pak & Tang, 1933], but it increases the action, of small doses at any rate, on the nictitating membrane [Bacq, 1936; Bülbring & Burn, 1937].

The first two of these actions of ephedrine resemble the action of eserine on the pupil in the fact that they are diminished by degeneration of the nerves and can be explained on the theory that they are due to inhibition of an enzyme which would otherwise have destroyed the chemical transmitter. The action of ephedrine on the nictitating membrane is clearly different since it is increased, like that of adrenaline, by nervous degeneration. Ephedrine probably causes the nictitating membrane to contract in exactly the same way that adrenaline does. This conclusion is not necessarily inconsistent with the theory that the potentiation by ephedrine of the action of adrenaline on the nictitating membrane is due to the inhibition of amine oxidase.

Further evidence comes from studies of the actions of substances allied chemically to ephedrine. Munch & Hartung [1929] studied the potentiation of the action of adrenaline on dog's blood pressure by compounds with the general formula $R_1.C_6H_4.CHOH.CHR_2.NH_2$. The substance in this series in which R_1 is H and R_2 is CH_3 differs from ephedrine only in the absence of a methyl group attached to the nitrogen. Potentiation occurred when R_2 was CH_3, but not when it was H, C_2H_5 or C_3H_7. R_1 was usually H, but might be OH or CH_3 without loss of the potentiation. Tainter [1931] observed potentiation with $(OH)_2.C_6H_3.CHOH.CHCH_3.NH_2$ (corbasil). These results are consistent with those of Blaschko *et al.* [1937*b*], who studied a more diverse series of substances and found that the presence of CH_3 in the position of R_2 conferred immunity to amine oxidase. The evidence is incomplete, but the fact that substances with CH_3 in the position of R_2 have been found to differ from allied substances both in their immunity to amine oxidase and in their potentiating action, supports the view that the potentiation is due to the enzyme becoming blocked up with substances which it cannot destroy.

While *l*-corbasil itself has a pressor action similar to that of *l*-adrenaline, *d*-corbasil has a much smaller pressor action, but when a large enough dose is injected the pressor effect is much more prolonged [Schaumann, 1931, 1937]. Since corbasil is immune to amine oxidase [Blaschko *et al.* 1937*b*] this enzyme is not the main cause of the short duration of the pressor action of the more active of these isomers. The duration of the action seems to depend rather on the dose injected, so that amines with low activity appear to have a prolonged action because larger doses are used and these large doses remain in the circulation for longer than the comparatively small doses of more active amines, which may produce a larger immediate rise of blood pressure. These facts are consistent with the view that the disappearance of the pressor action is mostly due to the diffusion of the amines out of the blood into the tissues. On the other hand, the fact that certain inhibitors of amine oxidase may cause some increase in the pressor action of adrenaline suggests that amine oxidase does play some part in removing adrenaline from the circulation. If it was the only factor the potentiation might be expected to be much more marked than it actually is.

All these actions of ephedrine can be attributed to the inhibition of amine oxidase, but can also be explained in other ways. They might be attributed, like those of cocaine [Cannon & Rosenblueth, 1937], to an increase in the permeability of the tissues. On this theory the potentiation would be due to easier access, and the increased yield on stimulation of the nerves would be due to easier escape, of the chemical transmitter from the tissues. A similar theory was advanced by Burn [1932], who explained the fact that the presence of adrenaline was necessary for the action of ephedrine by suggesting that ephedrine acted by liberating adrenaline from a local store which became depleted unless refilled by circulating adrenaline.

The potentiation might be due to selective paralysis of the inhibitor actions of adrenaline leaving the motor actions comparatively unaffected. This theory would be the opposite of that advanced by Dale to explain the action of ergotoxine [1906].

The effects might be due to the inhibition of some other enzyme which shared with amine oxidase the property of being inhibited by ephedrine.

Similar theories could be advanced to account for the actions of eserine, but there is no need to adopt any of them at present because there is no evidence against the theories which attribute the effects to the inhibition of choline esterase and amine oxidase. It is simpler to explain the actions of eserine and ephedrine in terms of their known effect on enzymes, which can be demonstrated *in vitro*, than to postulate new properties for them.

If this theory is true, the observation that ephedrine potentiates the action of adrenergic nerves provides direct evidence that both the substance liberated into the circulation by these nerves and the enzyme inhibited by ephedrine do really play an intimate part in the transmission of impulses.

Antagonism. The effect of high concentrations of ephedrine in diminishing the response to adrenaline can be explained on the theory that the ephedrine combines with the motor receptors and blocks them up [Curtis, 1929]. This theory is similar to the theory of substrate competition advanced above to account for the sensitization. Similar theories have been advanced to account for other specific antagonisms [Ringer & Morshead, 1880; Gaddum, 1936; Clark, 1937].

The fact that substances allied chemically to adrenaline may antagonize its action is well known [Loewe, 1927]. These other antagonisms may be accounted for by the same theory.

The immediate effects of ephedrine

Some of the immediate effects of an injection of ephedrine by itself are probably due to the inhibition of amine oxidase, and some of them are not. The evidence of Schaumann [1928] and Burn [1932] that ephedrine has practically no vasoconstrictor action on perfused tissues in the absence of adrenaline suggests that the vasoconstrictor action of ephedrine is entirely due to the inhibition of amine oxidase. This conclusion is confirmed by the experiments, discussed above, on the effect of the degeneration of adrenergic nerves. The evidence suggests that the effect of ephedrine on the pupil is also due to the same mechanism, but that the effect on the nictitating membrane is not. The diminishing effect on the blood pressure of successive doses of ephedrine (*tachyphylaxie*) may perhaps be due to blockage of the motor receptors by ephedrine, which thus excludes the chemical transmitter through whose preservation from the enzyme the earlier doses of ephedrine produce their effect.

SUMMARY

1. An improved method of perfusing the rabbit's ear is described. Stimulation of the sympathetic nerves in this preparation causes vasoconstriction and the liberation of a substance which can be detected by a colorimetric test for adrenaline.

2. Low concentrations of ephedrine sensitize the rabbit's ear, the cat's nictitating membrane, and the frog's heart not only to adrenaline, but also to the stimulation of adrenergic nerves.

3. Ephedrine increases the yield of the substance liberated by the nerves so that its properties can be more easily studied. It is not noradrenaline, epinine, corbasil or adrenalone, but may be adrenaline.

4. These actions of ephedrine are attributed to the inhibition of amine oxidase. This effect is compared with the inhibition of choline esterase by eserine.

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REACTIONS OF AVIAN MUSCLE TO ACETYLCHOLINE AND ESERINE

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The sensitivity of avian muscle to substances having nicotine-like actions has been known for many years. In its reactions to acetylcholine, avian muscle closely resembles normal amphibian muscle, and mammalian muscle during the course of degeneration of its motor nerve supply [Gasser & Dale, 1926]. Recent investigations have shown that normal mammalian muscle is not insensitive to acetylcholine, but that this substance, when suitably administered, can set up propagated contractions, arising from the motor end-plate [Brown *et al.*, 1936; cf. also Buchthal & Lindhard, 1937]. The application of similar methods to the study of frog's muscle and denervated mammalian muscle has shown that the reaction of these muscles to acetylcholine cannot be regarded as consisting only of a contracture proper, but that it consists of two phases, an initial outburst of propagated disturbances arising in the region of the motor end-plate, and a longer lasting contracture, during which propagated contractions cannot occur in the muscle [Brown, 1937*b*]. In the present investigation we have been able to demonstrate the same phases in the reactions of the normal and the denervated avian muscle.

In other respects a pharmacological study of avian muscle was desirable. Eserine has a very characteristic effect on mammalian muscle, converting the single response of the muscle to a motor nerve volley into a brief tetanus. In amphibian muscle, on the other hand, the effect of eserine is less certain, and it requires special methods to elicit the characteristic repetitive response as seen in the mammal [Cowan, 1936; Feng, 1937]. It was of interest, therefore, to observe the effects of eserine on the response to a single nerve volley of the bird's muscle, with its intermediate type of response to acetylcholine, and further to consider both of these in connexion with the peculiarities of neuro-muscular conduction in the bird, which we have earlier described [Brown & Harvey, 1938].

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METHODS

In these experiments we have used domestic fowls anaesthetized with "Pernocton". The choice and preparation of the birds for myographic recording, and the apparatus for recording and stimulating, have been described in a previous paper [Brown & Harvey, 1938]. As the anatomy of the fowl's gastrocnemius differs from that of the mammal, a modification of the usual method for close arterial injection into that muscle has been necessary. The nerve and blood vessels were exposed as previously described, and the lateral head of gastrocnemius was prepared for recording. The sciatic nerve was carefully dissected from the closely underlying artery and vein, and all branches of the nerve, other than the one supplying the lateral head of gastrocnemius, were cut. Immediately dorsal to the knee-joint, the sciatic artery and vein divide to form the anterior and posterior tibial vessels. The anterior tibial vein was tied and cut close to its junction with the sciatic vein and retracted by a thread on the cut end. This exposed the anterior tibial artery, into which the injection cannula was eventually inserted. All branches of this vessel were ligated and it was tied some 2 cm. distal to its origin. The branches of the posterior tibial artery and vein could now be identified, tied and cut, with the exception of those running into the base of the lateral head of gastrocnemius. The anterior tibial artery was now clamped, and a needle cannula was inserted into its distal end. When an injection was made, the circulation was stopped by clamping the main sciatic artery just proximal to its bifurcation, and a measured volume of fluid, usually 0·5 c.c., was rapidly injected from a syringe attached to the cannula. Injections of a suspension of Indian ink showed that such a volume reached the whole of the lateral head of the muscle, and did not spread into the surrounding tissues.

Acetylcholine chloride was used in freshly prepared solution in saline at *p*H 4. Control injections of the acid saline were usually without effect, though small responses may occur in the denervated muscle, as observed by Brown *et al.* [1936] in the denervated mammalian muscle. The action potentials resulting from the injection of acetylcholine were recorded with concentric needle electrodes, amplifier and oscillograph as previously described for mammalian and amphibian muscle [Brown, 1937*a*, *b*]. For the experiments on denervated muscle, the sciatic nerve was cut in the thigh, under Pernocton anaesthesia and with aseptic precautions. From 6 to 10 days were allowed for degeneration to proceed.

RESULTS

The effects of close arterial injection of acetylcholine

When administered by the close arterial route, acetylcholine causes muscular contractions in very minute doses. We have observed contractions equal in tension to the maximal motor nerve twitch from the injection of amounts as low as 0·05 μg. given in a volume of 0·5 c.c.—a concentration of 1 in 10^7 (Fig. 1). With doses of this order, the contraction is brief and, when recorded on a slowly moving surface, differs little superficially from the motor nerve twitch. In many respects it resembles closely a similarly evoked contraction of mammalian muscle, but differs from it in that there is always a slight prolongation of the contraction at the foot of the relaxation. As the dose is increased, the tension attained in the rising phase of the response increases up to about twice that of the twitch tension, but the most striking phenomenon is the increase, in both tension and duration, of the slow contraction which follows the initial, quick rise (Fig. 1).

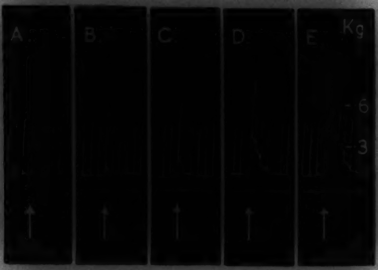


Fig. 1. Hen 1·5 kg. Maximal motor nerve twitches of normal gastrocnemius at 10 sec. intervals. At arrows, close arterial injection of A, 0·05 μg.; B, 0·1 μg.; C, 0·25 μg.; D, 0·5 μg. and E, 1·0 μg. acetylcholine, during intermission of one shock, and at C, two shocks, to nerve.

As will be seen in this figure, the smaller doses of acetylcholine do not reduce the visible response of the muscle to excitation through its nerve. With the larger doses there may be considerable depression of the twitch tension during the maintained contraction evoked by the injection

(Figs. 2 and 3). One phenomenon is very obvious in these records, and is even more apparent if the recording lever is watched during the response; namely the relaxation and recovery of the maintained tension, which follows the twitch in response to a nerve volley delivered during the maintained tension. This is evidently the phenomenon of "Durchreissen" which Riesser & Neuschloss [1921] described as occurring in frog's muscle, excited by a single shock during the prolonged response caused by immersion in acetylcholine solution.

Fig. 2. Hen 1·7 kg. Maximal motor nerve twitches of gastrocnemius at 10 sec. intervals. At arrow, close arterial injection of 10 μg. acetylcholine during intermission of one shock to nerve.

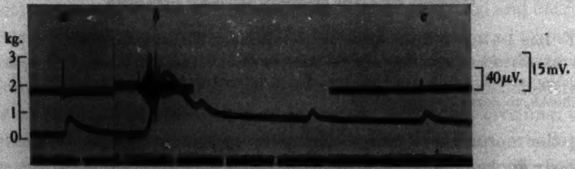


Fig. 3. Hen 0·9 kg. Isometric myogram and action potential record. (*a*) Maximal motor nerve twitch. (*b*) Injection of 20 μg. acetylcholine, during stimulation of motor nerve at intervals of 1 sec. Electrical record: (*a*) and (*c*) belly-tendon lead, (*b*) concentric needle electrodes. At blank space in record, amplifier switched back to belly-tendon lead. Time, 0·5 sec.

Degeneration of the motor nerve to avian muscle produces little qualitative change in the response to acetylcholine, but greatly increases the sensitivity of the muscle. Contractions of the same tension as the maximal, directly excited muscle twitch have been evoked with doses of acetylcholine between 0·0025 and 0·005 μg. injected into a volume of 0·5 c.c., i.e. in concentrations between 1 : 2×10^8 and 1 : 10^8. This represents approximately a tenfold increase in sensitivity, in comparison with that of the normal muscle. In some preparations we have determined the minimal dose necessary to produce a response. This may be as low as 0·00005 μg. in a concentration of 1 in 10^{10}. In determining these threshold doses care has been taken to exclude the apparent contraction produced by the sudden volume change in the muscle, and the occasional response which follows sudden injection of a large volume of acid saline. As mentioned above, the form of the response in denervated muscle differs little from that seen in the normal (Fig. 4). The only noteworthy difference we have observed is the tendency for the distinction between the sharp initial rise in tension and the slow subsequent maintenance of tension to be a little less evident in the denervated preparations. Directly excited muscle twitches are depressed during the slow relaxation, and this depression increases with increase in the dose.

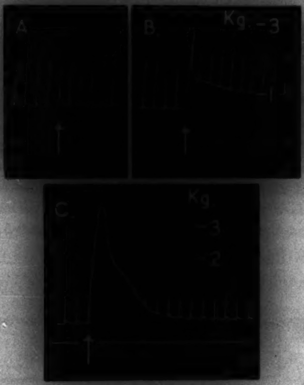


Fig. 4. Hen 1·3 kg. Maximal directly excited twitches of denervated (10 days) gastrocnemius. At arrows, close arterial injection of A, 0·0025μg.; B, 0·005μg., and C, 2·5μg. acetylcholine, during intermission of one shock.

Electrical phenomena

Mechanical records from denervated mammalian muscle show very clearly that the response to acetylcholine can be divided into two phases, a quick initial reaction and a much slower contracture [Brown, 1937*a*]. In frog's muscle the twofold character of the response is less obvious, and this applies also to the fowl's muscle. Electrical recording has, however, shown quite clearly that the response of avian muscle can similarly be divided into a "quick" phase, accompanied by oscillatory action potentials, and a "slow" component or contracture not caused by propagated disturbances in the muscle fibres. Fig. 5 shows the electrical and mechanical responses accompanying the injection of various concentrations of acetylcholine. With the smaller doses, the mechanical response has associated with it an outburst of electrical activity exactly like that in the mammal. As the dose is increased, the action potentials accom-

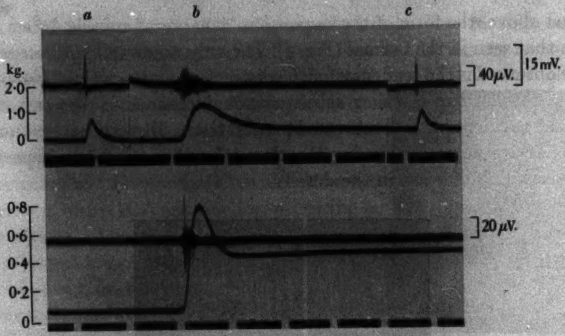


Fig. 5. Hen 1·5 kg. Myogram and action potential of gastrocnemius. Upper record, maximal motor nerve twitches, belly-tendon lead, (*a*) before and (*c*) 3 sec. after close arterial injection of 2·5μg. acetylcholine. In (*b*), response recorded with concentric needle electrodes. Lower record, response, in another experiment, of gastrocnemius to 5·0μg. acetylcholine. Concentric needle electrodes. Time 0·5 sec.

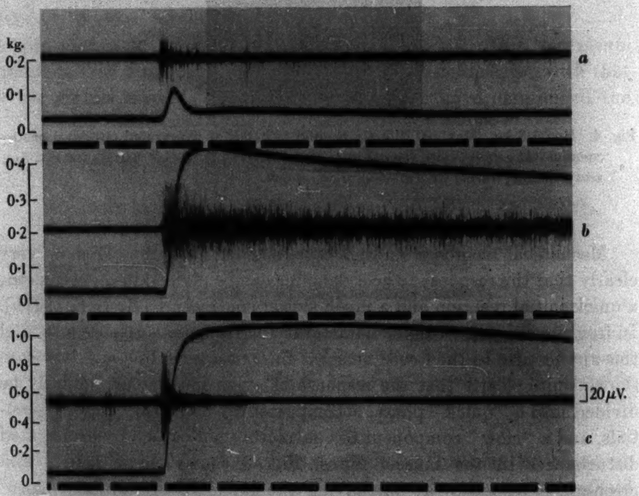


Fig. 6. Hen 1·3 kg. Myogram and action potentials of denervated (10 days) gastrocnemius, in response to close arterial injection of (*a*) 0·05μg., (*b*) 0·25μg. and (*c*) 2·5μg. acetylcholine. Concentric needle electrodes. (*c*) Shows spontaneous discharge. Time 0·5 sec.

panying the quick rise in tension are initially increased in magnitude and frequency, but the activity ceases abruptly as the tension crest is attained. With doses approaching the maximal, the oscillatory potentials are confined to the earliest rising phase of the response, and during the maintained tension the muscle is electrically "silent" (Fig. 5). Almost identical responses can be recorded from denervated muscle (Fig. 6). This figure also shows a point of considerable interest. In (*b*) and (*c*) are shown consecutive injections of 0·25 and 2·5μg. It will be observed that the mechanical changes are almost identical in form; in (*b*) there is a prolonged electrical activity, whereas in (*c*) the response is almost entirely without electrical accompaniment. This figure demonstrates the impossibility of deciding, from mechanical records alone, the nature of the response to acetylcholine. In the course of studying the responses of the muscle to very small concentrations of acetylcholine we frequently observed that a considerable mechanical response could occur without the appearance of any appreciable electrical activity, when concentric needle electrodes were used. This finding was surprising, in that previous work has shown that the contraction from small doses in denervated mammalian and in frog's muscle is always associated with oscillatory potentials, and that only higher concentrations evoke the contracture proper. The difficulty was eliminated, however, by finding that oscillatory potentials could readily be recorded in these small dose responses, if the leads were taken from belly and tendon. It would appear that the response to small doses is confined to groups of fibres scattered throughout the muscle, and that the localized lead provided by the needle electrodes may not include a sufficiently wide area to show the activity of a number of scattered fibres involved in a response.

Effect of eserine on the response to acetylcholine

As might be expected from previous work on mammalian muscle, eserine has a relatively small effect on the tension maximum of the response to close range injection of acetylcholine. Increases up to 50 % have been observed. Its most striking effect is in the prolongation of the slow relaxation (Fig. 7). This action is almost identical with the effects observed in denervated mammalian muscle.

Effects of eserine on response to nerve stimulation

The effects of eserine on the response of avian muscle to excitation through its nerve are weak and uncertain in comparison with the corresponding effects seen in the mammal. No obvious potentiation of the

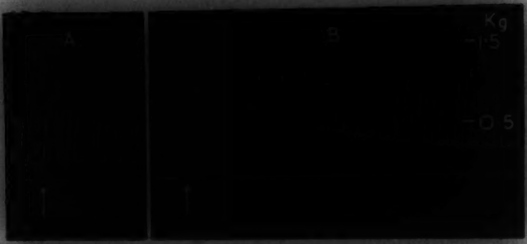
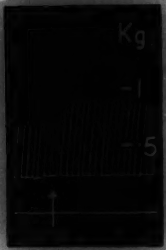


Fig. 7. Hen 1·6 kg. Maximal motor nerve twitches of gastrocnemius at 10 sec. intervals. At arrows, close arterial injection of 2·5 μg. acetylcholine. Between A and B, 25 μg. eserine was injected into the muscle.



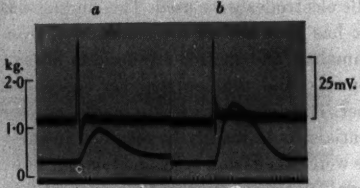


Fig. 8. Fig. 9.

Fig. 8. Hen 2·0 kg. Maximal motor nerve twitches of gastrocnemius at 10 sec. intervals. At arrow, close arterial injection of 50 μg. eserine.

Fig. 9. Hen 1·5 kg. Myogram and action potential (belly-tendon) of gastrocnemius in response to single maximal motor nerve volleys, (*a*) before and (*b*) 5 min. after close arterial injection of 50 μg. of eserine. Time, 10 msec.

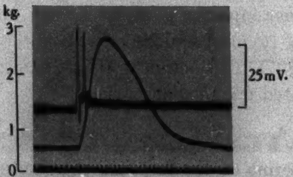


Fig. 10. Same experiment as Fig. 9. Response of gastrocnemius to two motor nerve volleys 20 msec. apart, after close arterial injection of 50 μg. of eserine. Time, 10 msec.

twitch tension has been observed when eserine has been given intravenously in doses from 0·4 to 1·0 mg. per kg. and with frequencies of nerve stimulation ranging from 1 to 6 per min. When the eserine has been given by local arterial injection into the muscle, more positive effects have been observed. Doses of 25–50 μg. have produced increases in twitch tension up to 90 % (Figs. 8 and 9). The most favourable conditions for eliciting the potentiation are produced when the frequency of the stimulation is kept as low as 1 in 10 sec., or even lower (1 in 30 sec.), and when the nerve has been stimulated with a tetanizing current for three or four periods of 10 sec. each, shortly before the eserine is given. The increase in twitch tension lasts approximately 5 min., and a subsequent dose, given after the first effect has passed off, can cause another increase in twitch tension. The greater potentiations are usually accompanied by a small but evident repetitive response of the muscle fibres to the single nerve volley (Figs. 9 and 10). However, we have frequently observed small degrees of potentiation to occur without there being any significant repetition.

Effect of two maximal nerve volleys in muscle under eserine

In mammalian muscle fully treated with eserine, a single maximal nerve volley produces a repetitive discharge of the muscle fibres which lasts at least 30 msec., and a second volley set up during this period is without visible effect. The response of avian muscles to similar excitation is quite different. After eserine, a second volley produces a response as soon as it is timed to fall outside the refractory period of the neuromuscular system (Fig. 10). The action potential of the response to such a second volley shows less repetitive activity than that of the response to the preceding volley. The most striking effect of eserine, however, is the reversal of the relative sizes of the first and second action potentials in response to a pair of motor nerve volleys. In a previous paper [Brown & Harvey, 1938] we have shown that, normally, a single nerve volley fails to excite some 25 % of the fibres of a bird's muscle, but leaves behind it, for 150 msec., a period of raised excitability of the motor end-plates. This period of raised excitability enables a second volley to excite additional fibres, and, consequently, to evoke a greater action potential. After the administration of eserine, the relation of first to second response is reversed. The first electrical response to a pair of volleys after eserine is greater in amplitude than the second response, and greater than the first response to a pair recorded before eserine (Table I). This would appear to show that, after eserine, a single volley can excite more muscle fibres than a single volley in normal muscle. In our previous paper we have shown

TABLE I.

Single responses

Before eserine		After eserine	
Tension kg.	Action potential mV.	Tension kg.	Action potential mV.
0·62	32·7	1·03	36·2
0·63	33·7	0·95	35·3

Mean increase in tension 57 %
„ „ „ action potential 8 %

Second electrical response as percentage of first electrical response with pairs of volleys at different intervals

Before eserine		After eserine	
Interval msec.	%	Interval msec.	%
30	113	26	84
51	111	50	95
92	105	92	95

that a similar change in the response to a single volley is produced, in the bird's muscle, by a brief tetanization of the motor nerve. After a tetanus, the first response of a pair involves approximately all the fibres of the muscle; the second response of a post-tetanic pair, although apparently still maximal, being associated with a reduced action potential. The same applies to the second response after eserine; it is reduced from 5 to 15 % below the level of the first response (Table I and Fig. 11).

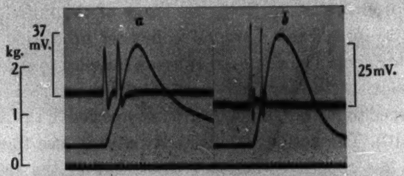


Fig. 11. Hen 1·5 kg. Myogram and action potential of gastrocnemius in response to pairs of motor nerve volleys (*a*) before eserine, 30 msec. apart, and (*b*) 5 min. after close arterial injection of 50 μg. of eserine, 25 msec. apart. Time, 10 msec. The amplification is greater in (*b*) than in (*a*).

Effects of tetanic stimulation after eserine

Mammalian muscle treated with eserine is characteristically unable to maintain its tension during a tetanus of a few seconds duration [Briscoe, 1936]. A tetanus produces, in addition, a prolonged subsequent depression of the responses of such a muscle to single maximal motor nerve volleys, an effect which can be imitated with considerable exactness by the arterial injection of acetylcholine [Bacq & Brown, 1937]. These effects are not normally seen in fowl muscle. The response to occasional tetani, interspersed among single volleys, is certainly not depressed by eserine; in fact, when any change is observed, it is usually in the direction of enhancement.

Fig. 12. Hen 1·6 kg. Maximal motor nerve twitches of gastrocnemius at 10 sec. intervals, after close arterial injection of 25 μg. of eserine. At arrow, maximal motor nerve tetanus at 50 per sec. for 20 sec.

The electrical response accompanying a tetanus after eserine differs little from that of normal muscle. The ratio of the amplitude of its first two action potentials may be reversed, as is to be expected from our observations on two volleys, and the first few responses may show a little repetitive discharge. The subsequent potentials are normal. A tetanus in fowl's muscle after eserine does not produce depression of the response to subsequent single volleys (Fig. 12); in fact, the usual post-tetanic potentiation of the twitch tension is still in evidence. Under special conditions, however, some degree of depression of the tetanic response can be demonstrated. Thus, if a muscle is tetanized for periods of 2 sec. at intervals of 1 min., the responses after eserine are slightly, but significantly depressed (Fig. 13). With this type of stimulation, before eserine, an injection of acetylcholine is not followed by a depression of the response to nerve excitation [cf. Gasser & Dale, 1926]. If, however, the acetylcholine is injected after eserine, subsequent responses to tetanic nerve stimulation are depressed, in exactly the same way as they are in mammalian muscle stimulated with single shocks (Fig. 13). We have occasionally observed after eserine a small and inconstant post-tetanic "contracture" lasting for some 10–20 sec., and producing little change in the twitch tension. We have not observed a similar post-tetanic contracture in normal muscle, stimulated either directly or through its nerve. Denervated muscle, however, when tetanized directly, shows a very characteristic post-tetanic contracture, which differs in many respects from that observed in the muscle under eserine (Fig. 14). When the denervated muscle has almost completely relaxed on the cessation of the faradic stimulation, the tension quite suddenly rises again, to reach a crest some 10 sec. after the initial relaxation, and then slowly declines. The post-tetanic contracture in eserinized muscle, on the other hand; is much more of the nature of a delayed relaxation. It may be noted that eserine has no effect on the tension response of denervated muscle to single stimuli, and does not obviously affect the post-tetanic contracture. The post-tetanic contracture of denervated muscle can be abolished by the arterial injection of atropine in doses of 100 μg., an observation reminiscent of the effects of atropine on the neuromuscular contracture [Bremer, 1932] and on the acetylcholine contracture in frog's muscle [Brown, 1937*b*]. Atropine, given arterially in doses up to 1 mg., is without significant effect on the other reactions of avian muscle.

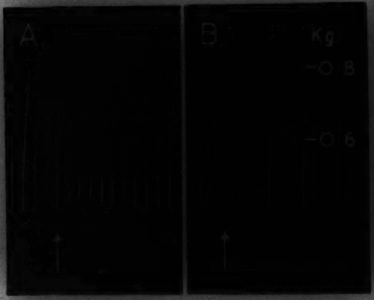


Fig. 13.

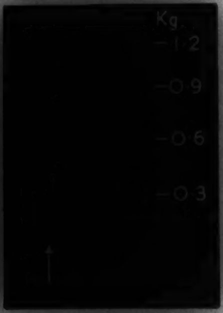


Fig. 14.

Fig. 13. Hen 2·5 kg. Maximal motor nerve tetani of gastrocnemius at 50 per sec. for 2 sec. at intervals of 1 min. At arrows, close arterial injection, in A, of 50 μg. of eserine; in B, of 5 μg. of acetylcholine 15 min. later.

Fig. 14. Hen 2·0 kg. Maximal directly excited twitches of denervated (6 days) gastrocnemius at intervals of 10 sec. At arrow, maximal tetanus at 50 per sec. for 3 sec.

DISCUSSION

We have found that avian muscle is considerably more sensitive to injected acetylcholine than mammalian muscle. If the amounts of acetylcholine required to produce a response equal in tension to the maximal motor nerve twitch are compared (0·05 μg. in the fowl and 2·5 μg. in the cat's gastrocnemius), the fowl's muscle appears to be some fifty times more sensitive, but precise comparisons are naturally impossible. The increase in sensitivity produced by denervation of fowl's muscle is of the same order as that produced in frog's muscle [Brown, 1937*b*], i.e. about ten times, which is much less than the approximately thousandfold increase which follows denervation in the mammal [Brown *et al.*, 1936]. This enormous increase in the sensitivity of mammalian muscle is accompanied by the development of a new feature of the response—the contracture. Avian and amphibian muscle, on the other hand, normally give a dual response to acetylcholine, a quick phase accompanied by oscillatory potentials and a contracture, not associated with rapid potentials. Denervation merely increases slightly the general sensitivity to acetylcholine, and slightly exaggerates the element of contracture.

The dual character of the response of denervated mammalian muscle, when acetylcholine is given by the close arterial route, is usually fairly obvious in mechanical records. Such clear distinction between the two phases of the mechanical response is often absent in amphibian muscle and in avian muscle, particularly after denervation. Raventos's [1937] claim that small doses of acetylcholine in frog's muscle produce a contracture, which passes, as the dose is increased, to a "quick" contraction, followed by contracture, is based on mechanical records alone, and is therefore inadmissible. Different doses of acetylcholine may produce almost identical mechanical responses, but, as shown in Fig. 6, one may be a prolonged tetanic contraction, whereas the other may consist of a brief tetanic outburst, followed by a genuine contracture. We are convinced that the first action of acetylcholine, in whatever dose it is applied to mammalian, avian and amphibian muscle, is the setting up of propagated contractions. In the absence of electrical records, the effect of an injection of acetylcholine on the response of a muscle to single or tetanic stimuli, applied directly or through the nerve, can give some indication of the nature of the contraction, since the presence of a contracture in the muscle fibres prevents the propagation of normal contractions. This is well illustrated in Fig. 6*a*, *b* of Gasser & Dale's [1926] paper, in which they show that an injection of acetylcholine produced in a normal avian muscle little effect on subsequent brief tetani, whereas a similar injection after denervation of the muscle profoundly depressed subsequent responses to tetanic stimulation. The responses to nerve volleys, falling during the tetanic phase of responses to acetylcholine, are not depressed, except in so far as they fall among fibres many of which may be in some stage of their refractory periods. The records which are difficult to interpret are those in which, after small doses of acetylcholine, subsequent single twitches arise from a slightly raised base-line, and may show a tension maximum above that of the preceding twitches. The electrical record in those instances shows clearly that such responses are, in fact, to some degree depressed (Fig. 5), and are superimposed on a small persisting contracture, involving only some fibres of the muscle. Using these criteria, we are doubtful whether an injection of acetylcholine ever, in normal avian muscle, produces a response which is purely of the propagated type, such as occurs in mammalian muscle; some small element of contracture appears always to be present.

Van Dijk [1936] has suggested that the response of avian muscle to acetylcholine is due to the contraction of one special fibre component—the intrafusal fibres of the muscle spindles. Our own evidence gives no support to this hypothesis. The contracture after a fairly large dose of acetylcholine completely abolishes the response of the muscle to nerve stimulation (Fig. 2). If van Dijk's conception is to hold, we must, therefore, postulate either that a motor nerve volley never excites any fibres but those within the muscle spindles, or that contracture of the intrafusal fibres prevents the contraction of the remainder of the muscle. A further, and we believe conclusive objection to van Dijk's theory is the fact that the external ocular muscles of the cat, which contain neither muscle spindles nor intrafusal fibres [Sherrington, 1894], are exceptional among the voluntary muscles of that animal, in contracting readily in response to acetylcholine given intravenously. We have no reason to doubt that differences in responsiveness to acetylcholine exist among the fibres of a large muscle, but, in our opinion, a sufficiently large dose of acetylcholine can produce both propagated contractions and contracture in all the fibres of a bird's muscle, under suitable conditions of application.

The effect of eserine on avian muscle differs from its effect on mammalian muscle in the following respects:

(1) A small degree of potentiation can occur in the absence of detectable repetitive discharge of the muscle.

(2) When repetitive discharge occurs it is of small dimensions and transient, and can be regularly produced only by previous tetanization of the muscle.

(3) A second volley set up during the repetitive discharge evokes a response only slightly less than the normal second response.

(4) The response to tetanic stimulation is not ordinarily depressed by eserine, and a tetanus does not depress subsequent responses to nerve stimulation.

The phenomenon of increased tension in the absence of repetitive discharge is readily understandable if the conditions of neuromuscular conduction in normal avian muscle are considered. In a previous paper [Brown & Harvey, 1938] we have shown that normally a single maximal nerve volley fails to excite all the fibres of a fowl's muscle. This submaximal effect of a single volley was found to be removed by the facilitatory effects of a preceding single volley, or in more lasting form by a preceding tetanus. Any procedure, therefore, which renders the transmission of the stimulus more effective, or which lowers the threshold of the muscle end-plates, will be followed by an increase in tension in response to single volleys. We have little doubt that the non-repetitive potentiation which may follow eserine is due to such an effect. Direct proof of an increase in the number of fibres responding would be given by the occurrence of an increase in action potential after eserine, and this we have been able to demonstrate (see Table I). Additional, indirect evidence that there is an increase in the number of fibres responding is provided by the observations on the ratio of first to second electrical response, when the muscle is excited by two rapidly succeeding nerve volleys. In normal muscle a second nerve volley excites more fibres than one immediately preceding it. After a brief tetanus the ratio of first to second electrical response is reversed, the first volley exciting all the muscle fibres and the second electrical response becoming less than the first, not because fewer fibres are contracting, but probably on account of its occurrence during the negative after-potential succeeding the first, maximal response. A similar reversal of the ratio of first to second response occurs after eserine. The increase in the size of the first response of a pair after eserine must be attributed to an increase in the number of fibres responding. The depression of the second response is probably, therefore, due to the same effects which are concerned in producing the depressed second response in a post-tetanic pair. When the first response of a pair after eserine shows repetitive discharge, another factor, refractoriness of some fibres, probably adds to the degree of this depression. Whether this effect of eserine, in increasing the number of fibres responding to a single volley, is attributable to a non-specific effect on cellular excitability, or to inhibition of esterase, must be left an open question.

Mammalian muscle treated with eserine responds to a single nerve volley with a tetanic outburst; the partially synchronized repetitive response of this can persist for some 30 msec., and the total duration of the activity following a single volley can be as long as 0·5 sec. This has been attributed to the persistence above threshold level of the acetylcholine liberated at the motor nerve terminals, and the long duration of the effect has led to the suggestion that the motor end-plate normally receives as a stimulus a greatly supraliminal amount of acetylcholine [Brown, 1937*a*]. In avian muscle, eserine can only produce repetitive discharge when the end-plate threshold has been lowered by previous tetanization of the nerve, and, when produced, it is small and transient. Avian muscle is more sensitive to applied acetylcholine than mammalian muscle, and consequently a higher threshold of the motor end-plate cannot be invoked to explain the difference. A possible explanation, which is compatible with many features of neuromuscular conduction in the bird, is that a single nerve impulse liberates an amount of acetylcholine which only just attains a liminal value for some fibres, and is below it for others.

This hypothesis provides an explanation of the failure of a single volley to excite all the fibres of the muscle, and of the very slight direct action of eserine. Tetanic stimulation of the motor nerve of a mammalian muscle under eserine produces a long lasting depression of the response to single volleys, an effect which can be closely imitated by injection of acetylcholine, and which has been attributed to accumulation in the muscle of the acetylcholine liberated at the nerve endings. This depressant effect of a tetanus is characteristically absent in avian muscle, and only appears when eserine is given during short periods of repeated tetanization. Acetylcholine injected into avian muscle under eserine produces a long lasting subsequent depression of nerve twitches, by virtue of the contracture which it causes. A contracture may occasionally follow prolonged tetanization of avian muscle under eserine, but it is small and transient, and produces little effect on twitch tension. These effects of tetani in avian, as compared with mammalian muscle, are not only compatible with the idea of a small liberation of acetylcholine at the avian motor nerve ending, but are difficult to explain on any other hypothesis.

SUMMARY

1. The responses of normal and denervated avian muscle to acetylcholine and eserine have been studied by the method of close arterial injection.

2. The injection of acetylcholine evokes a double mechanical response consisting of a quick initial response followed by a contracture, which becomes more evident as the concentration of the drug is increased.

3. The quick response to injected acetylcholine is associated with an outburst of action potentials, which are stopped by the onset of contracture, during which no rapid action potentials are present.

4. Denervation increases the sensitivity of the muscle about ten times.

5. The response of denervated muscle is qualitatively little different from that of the normal, although the contracture is more in evidence.

6. Eserine increases the quick phase of the response to acetylcholine and greatly prolongs the contracture.

7. It produces a potentiation of the tension response to maximal motor nerve stimuli, smaller, however, and more transient than that in mammalian muscle.

8. The potentiation is due to an increase in the number of muscle fibres responding to a single nerve volley and to a small repetitive discharge of the muscle.

9. The responses of a muscle under eserine to single maximal nerve volleys are not depressed by the interpolation of a tetanus.

10. It is suggested that these differences between avian and mammalian muscle can be accounted for by assuming that the amount of acetylcholine, liberated by a single impulse at a motor nerve ending in avian muscle, only just reaches the threshold level in the case of most fibres, and is subliminal for some.

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THE CHLORIDE CONTENT OF BLOOD SERUM AND AQUEOUS HUMOUR. ITS RELATION TO GLAUCOMA AND TO THE FORMATION OF INTRA-OCULAR FLUID

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In a recent paper it was shown that the osmotic pressure of aqueous humour was higher than that of blood serum by an amount corresponding on the average to 0·004 *M* sodium chloride [Benham *et al.* 1938]. This result was obtained from experiments on man and on dogs using only local anaesthesia and was the reverse of that previously reported, wherein the osmotic pressure of the blood serum was found to be greater than that of the aqueous humour by an amount compatible with the formation of the latter by a process of dialysis. Thus Duke-Elder [1927] had determined the osmotic pressure of the aqueous humour of rabbits by direct measurement and found it less than that of the blood, and this seemed to be verified later for cats using the more accurate technique of the vapour pressure method [Benham *et al.* 1937]. In this earlier work, however, general anaesthesia was employed, and herein lay the discrepancy, for it has been shown that the effect of general anaesthesia in animals is to raise the osmotic pressure of the blood from a normal value below that of intra-ocular fluid to a value above it, thereby reversing the relationship. This effect is due partly to muscular movements associated with anaesthesia and partly to the effect of ether on the acid-base equilibrium. The effect is temporary, for after some hours of anaesthesia the osmotic pressure of the aqueous humour becomes again greater than that of the blood serum. It will be appreciated, however, that since aqueous humour is normally at a higher osmotic pressure than the blood serum, it cannot be a dialysate, for by the theory of dialysis the osmotic pressure of the parent fluid (the blood) should be greater than that of its dialysate by the osmotic pressure of the plasma proteins plus the small pressure difference existing by virtue of the unequal distribution of the electrolytes. Realizing the important role played by chloride ions in the fluid interchange in the body it is reasonable to expect that a considerable part of the higher osmotic pressure of aqueous humour might be derived from an excess of chloride in the intra-ocular fluid. Furthermore, it is possible that the distribution of chloride may be an important factor in the pathologically raised intra-ocular pressure of glaucoma. Using cats under ether anaesthesia Davson *et al.* [1936] claimed that the serum-aqueous partition of chloride was in accordance with that required by a Gibbs-Donnan equilibrium. In their work, the anterior chamber of the eye was emptied by suction, thereby incurring the risk of transudation from the vessels of the iris, and some of the results were derived from pooled samples of aqueous humour and serum. In view of what we now know of osmotic relationships it is important to determine the partition of chloride in man by an accurate ultra-micro method and under more carefully controlled conditions. The present investigation has a twofold purpose, to show (1) to what extent chloride is a factor in the maintenance of osmotic pressure within the eye, and (2) whether simultaneous determinations of chloride in serum and aqueous humour in glaucoma can throw any light on the etiology of that disease.

METHOD

Chloride was estimated in blood serum and aqueous humour by the silver iodate method of Sendroy [1937]. In this method the sample to be estimated is shaken with silver iodate powder, insoluble silver salt is precipitated, and the amount of iodate which goes into solution by the reaction is an accurate measure of the chloride present. The method possesses the advantage that the determination of the dissolved iodate affords a direct measure of the chloride, whereas measurements by difference, such as the Volhard titration, require two standard solutions and involve cumulative errors. Furthermore, the iodate functions as a hexavalent oxidizing agent in the oxidation-reduction reaction by which it is measured and thus 1 equivalent of chloride yields 6 equivalents of iodate to be measured. These 6 equivalents are measured titrimetrically by reaction with KI as 6 equivalents of free iodine. Thus this method is eminently suitable for an investigation of this kind, having in mind the small amount of aqueous humour ordinarily obtainable from human eyes. All determinations were carried out on 0·05 ml. of material with an accuracy of ±0·4%.

Experiments on man. Samples of blood and aqueous humour were obtained from patients with chronic glaucoma and from a control group of non-glaucomatous subjects. Since all the cases were hospitalized it was possible strictly to control factors such as exercise or the ingestion of food and fluid which would be likely to affect the blood-aqueous equilibrium. A small sample of aqueous humour, about 0·1 ml., was obtained by passing a fine hypodermic needle to which was attached a capillary tube, obliquely through the limbus of the cocainized eye. Blood was withdrawn anaerobically and without stasis from the antecubital vein after the temperature of the arm had been brought to 45° C. The results of determinations are given in Table I. They comprise a study of six cases of glaucoma and eight subjects selected as controls. The values are expressed as millimols per litre.

TABLE I

Case no.		mM. chloride (aqueous humour)	mM. chloride (serum)
1	Chronic primary glaucoma	129·4	110·5
2	Chronic primary glaucoma	128·1	108·1
3	Chronic primary glaucoma	127·0	107·4
4	Chronic primary glaucoma	125·3	105·6
5	Chronic primary glaucoma	123·5	105·1
6	Chronic primary glaucoma	124·8	102·3
	Average for six glaucoma cases	126·3	106·5
7	Intra-ocular tumour	125·9	102·5
8	Intra-ocular tumour	121·0	108·6
9	Congenital cataract	122·4	102·6
10	Aphakia	123·7	105·3
11	Aphakia	128·5	104·5
12	Aphakia	125·7	104·8
13	Congenital cataract	124·0	106·2
14	Aphakia	124·1	104·6
	Average for eight controls	124·3	105·1

It will be seen that there is no significant difference in the distribution of chloride between the control and the glaucoma group. Since the ocular tension in this series of fourteen subjects ranges from 20 mm. Hg to 70 mm. Hg (Schiötz) it is apparent that the intra-ocular pressure cannot be a simple function of chloride content. The significance of the results lies in the amount of the difference between blood-serum chloride and aqueous-humous chloride in any one subject. This difference amounts in round figures to 20 mM. and is of the same order as that found between serum and cerebrospinal fluid.

Experiments on dogs. Dogs were trained to permit the withdrawal of aqueous humour under local anaesthesia. Blood was obtained anaerobically by arterial puncture. The results of simultaneous determinations on blood and aqueous humour for ten dogs are shown in Table II.

There is more variation between individual values for the experiments on dogs, and this is attributable to the fact that the strict regimen as

TABLE II

Dog no.	mM. chloride (aqueous humour)	mM. chloride (serum)
1	130·1	106·0
2	124·3	107·4
3	127·6	113·0
4	125·7	112·6
5	129·1	104·0
6	128·1	111·4
7	125·6	108·7
8	128·6	112·7
9	127·1	107·5
10	130·4	107·9
Average	127·6	109·1

regards exercise, food and fluids, possible with co-operation in human patients, was not attempted for these animals. However, the trend is in the same direction, the average difference between aqueous humour and serum being 18·4 mM. compared with 19·3 mM. for man.

DISCUSSION

Van Slyke [1926] has shown that human serum in equilibrium with its dialysate should give a distribution ratio for $\frac{\text{dialysate}}{\text{parent fluid}}$ for chloride ions of 1·04. This ratio was found by Hastings *et al.* [1927] to hold good between ascitic or oedema fluid, and serum. If we now take the mean values for the control group of Table I and express them in terms of millimols per kg. of water they become 125·5 and 111·4 for aqueous and serum respectively, compared with 114·3 and 110·3 for ascitic fluid and serum found by Hastings and co-workers. The distribution ratio

$$r=\frac{\text{aqueous chloride}}{\text{serum chloride}} \text{ has a value of } \frac{125{\cdot}5}{111{\cdot}4}=1{\cdot}13,$$

against a predicted value of 1·04 on the basis of Gibbs-Donnan equilibrium. A deviation from Donnan's law for any substance involves a free energy change. This free energy change dF may be calculated approximately from the equation $dF=NRT\ln\frac{C\text{ aqueous humour}}{C\text{ serum}}$, where N is the number of mols of a substance present in the arbitrary quantity of aqueous humour, R is the gas constant, T the absolute temperature and C concentration. On this basis, assuming that activity of chloride ions equals their concentration, the minimum amount of work necessary for the concentration of chloride in aqueous humour is approximately 6·4 cal./litre. The sum total of energy required to produce aqueous humour could be found by obtaining such values for all constituents (some of which, like urea and glucose, appear to be lower in the intra-ocular fluid and would hence have negative values) or by calculations based on total osmotic pressure. It has been pointed out above that since aqueous humour has an osmotic pressure higher than its corresponding blood serum it cannot be a dialysate. The difference between the osmotic pressure actually observed and that predicted on the basis of a Gibbs-Donnan equilibrium can be converted into energy units and has been shown by Hodgson [1938], using a set of representative values, to yield the figure of 4·3 cal. for the production of 1 l. of intra-ocular fluid.

The conclusion to be drawn from this investigation is that the blood plasma and aqueous humour cannot be considered to be separated by inert membranes, which distribute ions in accordance with the requirement of a Gibbs-Donnan equilibrium. It seems likely that transudates such as ascitic and oedema fluids are dialysates, but in them the chlorine is much lower than in aqueous humour. The membranes in the eye, therefore, must possess the property of concentrating chloride in aqueous humour. As regards glaucoma the distribution of chloride does not differ from that of the controls, so that the very wide variations of intra-ocular pressure in the cases studied cannot have been caused by change in the concentration of this ion. It is possible that the high chloride of aqueous humour ensures the circulation of intra-ocular fluid and the maintenance of the intra-ocular pressure. Any interference with this flow will then cause a change in intra-ocular pressure, which, if resulting in an accumulation of fluid in the eye, would give rise to glaucoma.

SUMMARY

1. The chloride content of the aqueous humour and serum of man and dogs has been determined by the ultra-micro silver iodate method.

2. The values for a group of non-glaucomatous humans averaged, in millimols per litre: aqueous 124·3, serum 105·1.

3. There was no significant difference in the values obtained in cases of primary chronic glaucoma with high intra-ocular tension, where the average values were 126·3 for aqueous and 106·5 for serum.

4. Normal unanaesthetized dogs gave average values of 127·6 and 109·1 respectively.

5. The high values obtained for aqueous chloride show that there is no Donnan equilibrium for this ion between blood and aqueous humour, so that the latter cannot be regarded as a dialysate.

6. The concentration of chloride in the aqueous humour requires the expenditure of 4·3 cal. of energy per litre.

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WATER BALANCE AND BLOOD CHANGES FOLLOWING POSTERIOR PITUITARY EXTRACT ADMINISTRATION

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The production of anaemia in rabbits after the administration of posterior pituitary extract has been previously described by Dodds & Noble [1935], Dodds *et al.* [1935]. Following a subcutaneous dose of approximately 400 pressor units of an acetone picric acid extract or standard B.P. pituitary extract a number of animals developed a severe macrocytic anaemia. This usually appeared on the fourth to fifth day, with relatively greater reduction of cell count than haemoglobin. In view of the marked reticulocytosis that accompanied the anaemia as well as increased bile elimination, the anaemia was considered to be the result of increased blood destruction. McFarlane & McPhail [1937] produced a similar anaemia in guinea-pigs, but this did not appear for 10–15 days after injection. Hypophysectomy did not result in any marked changes in the red cell and haemoglobin content of the blood, nor influence the effect of posterior pituitary extract in producing anaemia. Gilman & Goodman [1935, 1936, 1937] fully confirmed the finding of anaemia in rabbits after "pituitrin" injection. They, however, criticized the tentative suggestion put forward by Dodds & Noble that blood destruction may be controlled by the posterior pituitary gland. From their observations that the serum osmotic pressure and red blood cell count showed a parallel decrease they believed that the anaemia was a result of water retention which caused blood dilution and thereby haemolysis. In further support of their conclusions they point out that oliguria followed "pituitrin" administration, and that a dry diet of oats with water *ad lib.* prevented the production of anaemia.

From a critical consideration of the papers of Gilman & Goodman a number of conclusions may be drawn. (1) The fact that the severe anaemia occurs only in a small percentage of rabbits has been overlooked. A large series is essential in order to determine what influence diet may have on the anaemia formation. (2) Fluid balance or blood-volume determinations were not made and therefore the implication that water retention occurs because of the oliguria is not justified. In a following paper it will be shown that the typical severe anaemia may occur in rabbits on a dry diet, and that no alteration in fluid balance or blood volume takes place.

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From a physiological viewpoint the suggestion that hydraemia, so severe as to cause blood destruction, would be produced in an animal from a normal fluid intake (if this did occur) with diminished urine output, is contrary to all scientific work on the control of blood volume and concentration. The blood volume and concentration would appear to be physiological constants which are most tenaciously maintained by the body. Even after the absorption of large amounts of water it has been difficult to demonstrate any consistent blood dilution. These experiments have been reviewed in papers by Chanutin *et al.* [1924], Greene & Rowntree [1927] and Findley & White [1937]. In animals which were subjected to forced water administration, Greene & Rowntree [1927] have shown that dogs may be given by mouth 5 % of the body weight of water every 30 min. for 5–8 hr. In these animals, which exhibit the symptoms of water intoxication, the average maximal fall produced in haemoglobin was from 100 to 83 % and in serum protein to 82 %, while the increase in plasma volume was from 100 to 114 %. Similar observations have been made by Underhill & Sallick [1925]. These changes must represent the maximal alterations which can be produced by water administration, with recovery of the animal. Even so, Greene & Rowntree state that "no evidence of haemolysis or of increased destruction of erythrocytes, such as haemoglobinuria, was observed". Gilman & Goodman [1937] found that the maximal changes following "pituitrin" administration were a reduction of haemoglobin from 100 to 33 % and of red blood count from 100 to 22 % (and a loss of body weight of some 400 g.), yet concluded that this anaemia is "a result of water retention causing blood dilution".

METHODS

In the following experiments the effects of large doses of posterior pituitary extract have been observed on rabbits and guinea-pigs. In fluid-balance determinations the animals were placed in metabolism cages and the urine collected and measured at 1 or 2 day intervals. In some cases a dry diet, consisting of oats, bran and chopped hay, has been used together with water *ad lib.* In order to obtain a rough idea of fluid intake, where a diet of high water content has been used, the animals were fed solely on carrots or swedes. Urine examination of specific gravity, albumin, sugar, blood, urobilin and microscopic findings was recorded. Blood examination consisted of red blood cell count, reticulocyte percentage and haemoglobin determination. The blood volume was measured using the vital red method [Peters & van Slyke, 1932]. A Leitz three-stage colorimeter was used for colour comparison, and serum taken previous to the dye administration was always used for the control. For blood volume it was found necessary to anaesthetize the rabbits with sodium amytal (75 mg. per kg. intraperitoneally), as preliminary observations without anaesthesia gave inconsistent results. B.P. pituitary extract has been administered by subcutaneous injection. In some cases the extracts used were prepared by the Kamm [1928] process, or by the acetone picric acid process [Dodds *et al.* 1934].

Results

Susceptibility. In rabbits the severe anaemia, as first described by Dodds & Noble [1935], has been produced in a relatively small number of animals. In all the experiments some sixty rabbits have received a dose of a pituitary preparation adequate to cause the severe anaemia. This, however, has occurred in only seven animals, or roughly in 11·5 % of cases. Some of these rabbits were receiving a wet diet, and one, the dry diet previously described. The anaemia has occurred in animals of both sexes and no particular strain or breed of rabbit has been used.

Slight or moderate anaemia (red cell count and haemoglobin more than 50 % of the original level) has been found to occur in nearly all the animals injected. A control extract (picrate) of anterior pituitary lobes was given in comparable doses to ten rabbits. Also, fourteen rabbits received injections of an extract (picrate) made from liver, kidney, and muscle. In these experiments there was no associated anaemia, and it is suggestive that the slight anaemia which follows posterior lobe extracts may be of significance. Liu & Noble [1938] have found that this type of anaemia also occurred after the injection of posterior lobe extracts directly into the renal artery. Under these conditions gastric haemorrhage could not be a factor in the anaemia.

Variations in extract administration. It was thought that the refractory animals might develop anaemia if the route of administration or the number of doses of the extract were altered. Pituitary extract has been administered intravenously to rabbits which failed to exhibit a severe anaemia after subcutaneous injection. Although doses as large as those used subcutaneously have been given over a short time it has not been found possible to produce anaemia in these animals. Other observations indicate that the intravenous route of injection does not materially increase the response to extracts. Thus, two rabbits of 3·3 and 2·8 kg. were injected through the ear vein with 100 and 130 pressor units of extract respectively. This was divided into three equal doses and given slowly at hourly intervals. No severe anaemia developed following this treatment or when somewhat larger doses were repeated 2 weeks later. Acute gastric ulceration has been shown to follow the administration of "pituitrin" by mouth in rabbits [Dodds *et al.* 1937]. In a few experiments it was found that no anaemia was associated with lesions produced in this manner.

Alterations in the spacing and size of dose given subcutaneously have been studied. Two animals which developed the severe anaemia from a single subcutaneous dose were allowed to return to normal and the injection then repeated. This was followed by a second period of severe anaemia. In another experiment two normal animals received 10 units, and two others 50 units three times a day for from 10 to 18 days without severe anaemia resulting. One of these animals at autopsy had a typical gastric ulcer. Large doses of 400 pressor units were injected subcutaneously into five rabbits at weekly intervals from 5 to 10 weeks. In no case did severe anaemia ensue. The effect of small doses of pituitary extract on the blood and fluid balance will be reported later in this paper.

Water balance with wet diet

In order to measure roughly the fluid intake rabbits were given 1 kg. of swedes daily as their only food. Urine was measured daily. Following the subcutaneous injection of 200 units of extract the urine excretion was markedly reduced usually for 5–6 days. During this time, however, the animals consumed very little food, and any positive water balance, if present, was not enough to be reflected in body weight. After such large injections nearly all animals developed severe diarrhoea which increased fluid loss. In Table I the alterations in fluid balance and body weight are shown in a typical experiment. The fall in urine output and food intake were maintained for 6 days, and then a rapid return to normal occurred. The blood showed a mild anaemia while the body weight remained unaltered. After a month's interval this animal received 200 units of extract given by mouth. In contrast to the previous antidiuretic action,

Table I. Effect of pituitary extract on fluid balance and blood of a rabbit

			Blood		
Days	Urine output c.c.	Swedes eaten g.	R.B.C. million/c.mm.	H.B. g./100 c.c.	Body weight kg.
1–7 (control)	304·2	698·5	5·63	11·5	2·06
200 units B.P. pituitary extract subcutaneously					
8	65	380	—	—	—
9	14	100	—	—	—
10	14	140	6·5	—	—
11	5	300	—	—	—
12	20	300	5·4	—	—
13	0	420	—	—	2·06
14	34	320	5·3	10·7	—
15	300	800	—	—	—
16	275	560	5·0	—	—
17	240	600	—	—	—
18	355	640	—	—	—
19	345	720	5·15	—	2·06

pituitary extract administered by mouth had no effect. For 4 days previous to the extract administration the food intake was 748 g. and urine output 354 c.c. The next two 4-day periods following the extract showed food intake 750 and 728 g. and urine output 331 and 331 c.c. respectively. In another experiment an attempt was made to decrease the urine excretion, but to maintain the animal's food intake. In a previous report [Dodds *et al.* 1937] it was shown that the antidiuretic activity of pituitary extract could be prolonged by the addition of zinc salts. A rabbit, therefore, was given small doses (5 units) twice daily of an extract in a 5 % zinc acetate solution. With this treatment it was found that the urine output was materially reduced, but the cabbage intake was only slightly lowered. A slight anaemia developed in this case and also a lowering of the body weight. Terminally anuria occurred and the food intake dropped without any increase, however, in the anaemia. These findings are recorded in Table II.

Table II. Effect of small doses of zinc pituitary extract on fluid balance and blood of a rabbit

Days	Urine output average c.c.	Cabbage eaten average g.	R.B.C. million/c.mm.	Body weight kg.
Control	245·0	500·0	5·6	2·18
5 units twice daily pituitary extract in 5 % zinc acetate				
1–4	14·5	202·5	5·5	2·18
5–8	61·7	265·0	5·25	2·10
9–12	71·2	377·5	4·05	2·10
13–16	40·0	286·5	3·70	2·08
17–19	0·0	78·3	4·00	2·06

A series of adult guinea-pigs were injected with 30 units subcutaneously. In Table III it is seen that the effect of this on urine output and food intake (carrots) was slight. None of these animals showed the typical severe anaemia, and it is apparent that a variation in the susceptibility of guinea-pigs occurs as well as in rabbits. These experiments made it appear unlikely that any increase in body fluid could account for anaemia production. Although the urine excretion was reduced there was an accompanying lowered fluid intake. Even when the food intake was maintained at as high a level as possible there was no evidence of increased blood destruction. In a further study blood volume has been measured as well as fluid balance.

TABLE III. Effect of pituitary extract on fluid balance and blood of guinea-pigs

	G. 71			G. 72		
Days	Urine average c.c.	Carrots average g.	R.B.C. millions/c.mm.	Urine average c.c.	Carrots average g.	R.B.C. millions/c.mm.
Control	120	200	6·3	65	145	6·35
30 units B.P. pituitary extract subcutaneously						
1	25	61	—	35	128	—
2	44	172	—	43	140	—
3	110	200	—	62	152	—
4–6	109	198	5·1	55	148	6·85
7–9	50	107	4·4	31	152	6·05
10–12	72	189	5·35	64	170	5·15
13–15	79	194	5·45	34	165	5·00

	G. 73			G. 75		
Days	Urine average c.c.	Carrots average g.	R.B.C. millions/c.mm.	Urine average c.c.	Carrots average g.	R.B.C. millions/c.mm.
Control	65	130	5·6	83	161	6·15
30 units B.P. pituitary extract subcutaneously						
1	31	140	—	48	120	—
2	54	180	—	53	144	—
3	72	200	—	48	157	—
4–6	68	200	5·1	80	185	6·3
7–9	68	200	5·7	86	200	5·75
10–12	84	195	6·5	60	193	5·35
13–15	99	200	4·5	65	184	5·25

Water balance and blood volume with a dry diet

In this series twelve rabbits have been given a dry diet, but with water *ad lib.* The fluid balance and the blood volume have been estimated as previously described. Four of these animals died shortly after the subcutaneous injection of 200 pressor units of extract, but the blood findings in the remaining eight are shown in Table IV. Rabbit 743 in this series

Table IV. Blood findings before and after 20 c.c. of posterior pituitary extract subcutaneously

Rabbit no.	Haemoglobin		R.B.C.		Reticulocytes		Minimum haemoglobin noted on day	Remark
	Before g./100 c.c.	After g./100 c.c.	Before millions/mm.^{3}	After millions/mm.^{3}	Before %	After %		
742	9·7	9·4	5·04	3·80	1·0	2·0	3	Survived
743	9·9	6·0	5·01	1·98	0·8	30·0	4	Death on 4th day
744	12·4	10·6	5·63	5·50	4·8	5·0	14	Survived
745	10·9	9·1	5·02	4·12	0·2	2·8	4	Death on 18th day of uræmia
1	9·9	9·7	5·06	4·50	5·2	4·6	3	Survived
3	10·9	8·3	5·20	3·45	1·4	8·0	7	,,
4	9·5	8·9	5·10	3·56	6·4	7·0	3	,,
10	11·4	8·5	5·50	4·88	1·0	6·0	13	Death on 13th day amytal anaesthesia
Average	10·7	8·8	5·20	3·97	2·6	8·2		

developed the typical severe anaemia and showed a reduction in haemoglobin from 9·9 to 6·0 g. and in red cell count from 5·01 to 1·98 millions on the fourth day after the injection. The other animals showed slight anaemia from the third to fourteenth day after the posterior pituitary extract administration. The average reduction of haemoglobin was from 10·7 to 8·8 g. and red cell count from 5·20 to 3·93 millions. Proportionally there seemed to be a slightly greater reduction in red cell count than in haemoglobin content. Reticulocytosis accompanied the anaemia. The water balance and the blood volume of these rabbits may be seen in Table V. For 10 days prior to injection, the daily average water intake for the series was 95 c.c. and the daily average urine output was 46 c.c.,

Table V. Data on water metabolism and blood volume before and after posterior pituitary extract injection

Rabbit no.	Water metabolism, average per day*						Blood volume per kg.		Body weight†	
	Water intake		Urine output		Balance					
	Before c.c.	After c.c.	Before c.c.	After c.c.	Before c.c.	After c.c.	Before c.c.	After c.c.	Before kg.	After kg.
742	82	73	60	24	22	49	58·6	66·5	2·44	2·54
743	132	34	71	76	61	−42	67·9	34·5	2·71	2·12
744	133	48	66	35	67	13	—	71·4	3·36	3·12
745	54	25	22	45	32	−20	—	82·8	2·60	2·12
1	102	103	47	41	55	62	78·5	77·6	2·80	2·86
3	94	86	38	66	56	20	89·4	79·6	2·60	2·08
4	74	78	34	32	40	46	84·2	69·7	3·06	2·74
10	88	49	33	25	55	24	79·7	75·0	2·32	1·96
Average	95	62	46	43	48	19				

* Average values of 10 days immediately before and after the injection.

† Body weight taken immediately before and 10 days after the injection.

leaving a positive balance of 48 c.c. As the animals remained fairly constant in weight the positive balance recorded represents largely the water lost through respiration and faecal elimination rather than an actual gain in body water content. After injection of the posterior pituitary extract the average daily water intake fell to 62 c.c. and urine output to 43 c.c. As the fall in water intake was much greater than that in urine output, the resulting balance of 19 c.c. after injection was much less than before. Two of the animals showed a negative balance. From

Table VI. Fluid balance and blood changes in R. 743 which developed severe anaemia

Days	Water intake average c.c.	Urine average c.c.	Haemoglobin g./100 c.c.	R.B.C. millions/c.mm.	Reticulocytes %	Blood volume c.c./kg.	Body weight kg.
0–20	132	71	9·9	5·0	1·5	68	2·7
20	200 units pituitary extract subcutaneously						
20–22	60	120	—	—	—	—	2·35
22–24	10	40	6·0	1·98	30·0	35	2·10
	Animal dead						

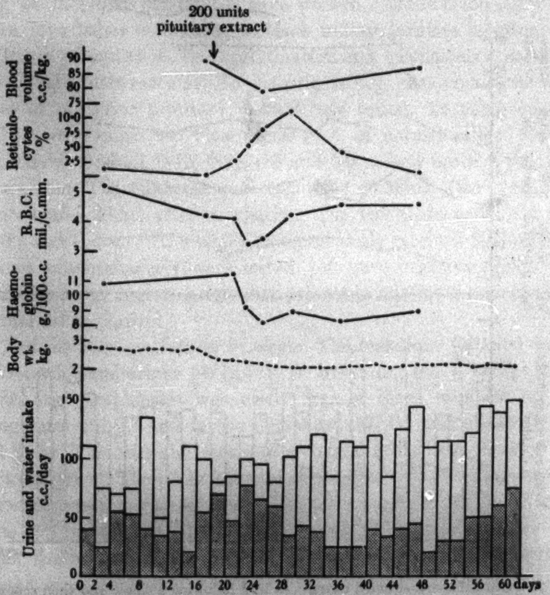

Fig. 1. Fluid balance and blood changes in R. 3 which developed moderate anaemia.

this it would appear that there was on the average a loss of body water after the administration of posterior pituitary extract. The loss of body water is further confirmed by the fact that most of the animals lost weight after the injection. Furthermore, the blood volume determinations (Table V) showed that most of the animals had a reduced blood volume after the injection.

From these observations it may be stated that in rabbits large subcutaneous doses of posterior pituitary extract resulted in slight to moderate anaemia in the majority of instances accompanied by loss of weight, decrease of blood volume and evidence of water loss. In R. 743 in which severe anaemia was produced, the weight loss was great, the blood volume decreased to half of its pre-injection level, and the water balance became negative. These findings are summarized in Table VI. The typical findings in an animal (R. 3) developing moderate anaemia is illustrated in Fig. 1.

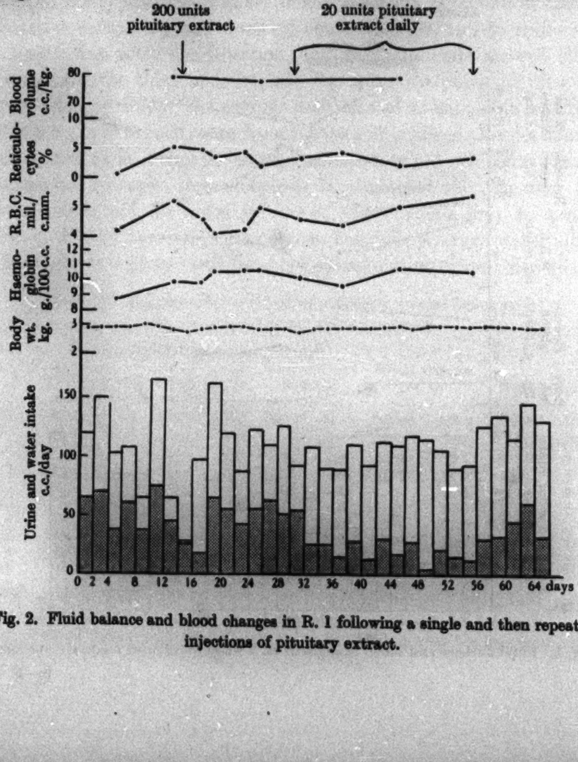

Fig. 2. Fluid balance and blood changes in R. 1 following a single and then repeated injections of pituitary extract.

Three of the rabbits (R. 742, R. 744 and R. 1), after recovery from the effects of the large subcutaneous dose of posterior pituitary extract, were given daily 2 c.c. (20 pressor units) of the extract subcutaneously for 26–39 days. No significant anaemia resulted in any of these animals. Urine output was suppressed during the period of pituitary extract administration and the water intake also decreased. The resulting water balance was not much greater than that in the control period, and the body weight remained stationary. The findings in R. 1, which is representative of the three chronically treated animals, are charted in Fig. 2.

Agglutination and haemolysis of red blood cells

In R. 743, which developed severe anaemia, it was noticed that the oxalated blood contained discrete particles which were identified under the microscope as clumps of agglutinated red cells. The plasma was intensely red, and on spectroscopic examination showed approximately 0·4 g. haemoglobin per 100 c.c. to be present. As the blood samples were taken into tubes containing isotonic sodium oxalate solution with a minimum of agitation, the agglutination and haemolysis observed were most likely intravascular. To investigate the phenomena the *in vitro* effect of posterior pituitary extract was tested. To tubes containing 0·05 c.c. of washed red blood cells 1 c.c. of normal saline, containing respectively 0, 0·05, 0·10, 0·25, 0·5 and 1·0 pressor units of the extract, were added. The mixtures were incubated at 37° C. Observations were made 3 and 20 hr. after incubation. The red blood cells of a control rabbit and those of R. 4 which had previously received 200 units of the extract subcutaneously were tested, but gave negative results. Slight haemolysis and agglutination were present in all the tubes, including the normal saline control.

Test for haemoagglutinin in serum. The technique followed was that of Donath-Landsteiner [Whitby & Britton, 1937], except that no guinea-pig complement was used. Sets of tubes were set up in the refrigerator (2° C.) and in the incubator (37° C.) and observed after 3 and 24 hr. The cells and serum of a control rabbit and those of R. 745 were examined. The latter animal had received 20 c.c. subcutaneously of posterior pituitary extract, developed slight anaemia and died of uraemia [Liu & Noble, 1938]. There was no gross agglutination of the cells in either the control or the pituitary extract injected rabbit's serum, although occasional small clumps of agglutinated cells were seen microscopically in both sera tested. The absence of clear-cut evidence of the presence of haemoagglutinin in the serum of the slightly anaemic rabbit does not exclude the possibility of its presence in animals made severely anaemic by posterior pituitary extract administration.

DISCUSSION

The present experiments confirm the previously reported fact that posterior pituitary extract in large subcutaneous doses was capable of producing anaemia in rabbits. Although severe anaemia was only occasionally produced, slight to moderate anaemia occurred in nearly all animals. The susceptibility of individual rabbits to the anaemia-producing effect of the extract appeared to vary, and was not related to the route of administration or size of dose. Small daily subcutaneous doses for long periods, or repeated intravenous injections were no more effective in producing severe anaemia than a single large dose subcutaneously. The cause of the severe and mild anaemia would therefore appear to differ. When the relatively small percentage of rabbits which develop the severe anaemia is considered it is probable that changes in diet do not alter the susceptibility. As it was seen in R. 745, it is possible to produce anaemia in animals fed a dry diet. The occurrence of anaemia in one animal out of eight is in keeping with the frequency found in animals on a wet diet.

From the fluid balance experiments with either a wet or dry diet it may be seen that pituitary extract caused a fall in urine output. The intake of fluid, however, always fell off in a parallel fashion. Even when special means were adopted to increase fluid intake over fluid output no evidence of severe anaemia was found. The blood volume, as estimated, did not increase after pituitary extract, and in an animal which died from the typical anaemia the blood volume was materially lowered.

No evidence was found in confirmation of the contention by Gilman & Goodman [1937] that "pituitrin anaemia in rabbits is the result of water retention causing blood dilution". All the described results are incompatible with such a suggestion, and it is obvious that water retention and blood dilution do not constitute an adequate explanation of the anaemia which may follow posterior pituitary extract injection in rabbits.

As shown by *in vitro* experiments, the extract probably does not have any direct destructive effects on the red blood cells. But, on the other hand, the fact that apparently autohaemoagglutination and haemolysis were present in one of the rabbits with severe anaemia suggests the possibility that the extract acts through the production of haemo-agglutinin and haemolysin. Subsequent experiments on other rabbits failed to demonstrate conclusively the presence of such agents, but further studies along these lines would be desirable.

SUMMARY

A small percentage of rabbits develop severe anaemia following subcutaneous injections of posterior pituitary extract, but mild or moderate anaemia (probably of a different origin) is frequently produced.

Various modifications of the route or frequency of injections failed to increase the susceptibility of the animals. Diet apparently was not an important factor in anaemia production.

A reduction in urine output followed injections of extract, but the fluid intake fell correspondingly. No positive increase in water balance or blood volume could be observed even though one animal displayed the typical anaemia.

The suggestion that pituitrin anaemia is the result of water retention causing blood dilution could not be supported from the experiments described.

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ACTION OF NICOTINE ON THE SPINAL CORD

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In previous papers [Schweitzer & Wright, 1937*b*, *c*, *d*, *e*, 1938] we examined the action of various autonomic drugs (adrenaline, acetylcholine and related substances, eserine and other anticholinesterases) on the central nervous system. In extension of this work we have studied the action of nicotine on the central nervous system, using the knee jerk as a typical spinal reflex. Dixon in his review in Heffter's *Handbuch* [1924] states that the main action of nicotine on the spinal cord is to produce convulsions. Langley & Dickinson [1890] give a detailed account of the action of nicotine in the frog, which is in agreement with the evidence of previous observers. There is initial excitement, followed by spasms, tonic rigidity and catalepsy. Finally the skeletal muscles become flaccid and reflex activity disappears. Langley [1901] noted that when a 1 % solution of nicotine is painted on to the spinal cord of the skate a violent discharge of impulses from the treated region takes place.

In the mammal, according to v. Praag [1855], Krocker [1868] and Truhart [1869], nicotine produces preliminary excitement, clonic spasms and twitchings of various muscles. Convulsions and opisthotonus were also noted. In the cat there is rigidity of the forelimbs (which is abolished by section of the peripheral nerves) and twitching may develop in the ears. Langley & Dickinson [1890] state that all these phenomena may be seen at times in anaesthetized animals, but to a lesser degree. Other observations, however, suggest that the excitatory actions of nicotine are not always easily evoked. Thus, Langley [1901] states that "in anaesthetized animals I have not found appreciable effects on applying nicotine locally to the spinal cord". On injecting 1 mg. of nicotine in 0·1 % solution peripherally into the 3rd lumbar artery of a deeply anaesthetized animal there was intense stimulation of sympathetic ganglia, but no muscular movements. In the mammal, Langley &

Dickinson [1890] "did not find any great difference between the time of disappearance of reflex contraction and muscular contraction produced by stimulating the motor nerve. As long as stimulation of the uncut sciatic nerve causes contraction of the leg on the side stimulated, it causes also reflex movements." **Langley** [1918–19] injected 2–5 mg. of strychnine into the anaesthetized cat. The subsequent injection of nicotine in doses up to 100 mg. in no case prevented clonic contractions in a leg with ligatured vessels. He concludes, therefore, "it follows that nicotine has no specific paralyzing action either on the anterior cornual cells or on the commissural cells on which strychnine is generally supposed to act". **Sollmann** [1937] summarizes the central action of nicotine by stating that it consists of stimulation followed by depression of the whole cerebrospinal axis from above downwards. Finally, it may be added that nicotine in strengths of 0·1–1 % directly applied to the spinal ganglia of mammals produces no stimulating or paralyzing action [**Langley**, 1901].

METHODS

Cats were used under chloralose anaesthesia (0·065–0·08 g. per kg. body weight). In a number of experiments decerebrate animals were used after 1 hr. or more had been allowed to elapse for the effects of the initial ether-chloroform anaesthesia to wear off. The knee jerk on one side and the response of the gastrocnemius muscle of the contralateral side to submaximal break shocks applied to its motor nerve were recorded, using our customary technique, which has been fully described in previous communications [**Schweitzer & Wright**, 1937*a–e*]. We used the "ischaemic hindlimb technique" to determine the extent to which nicotine produced its effect by an action on the central nervous system or on peripheral structures. In a number of experiments the carotid sinuses were denervated and the vagi cut. The injections of the drug were made into the jugular vein.

RESULTS

In the cat under chloralose anaesthesia, intravenous injection of nicotine depresses or abolishes the knee jerk (Fig. 1). The dose required to produce this effect varies considerably with the sensitivity of the preparation. The minimal dose which has produced a depression of the knee jerk is, in our experience, about 5μg. in animals weighing 3–4 kg. We have never noticed any increase in the knee jerk at any stage of the response, or with any size of dose. Doses which are too small to produce inhibition have no effect at all on the reflex. In some experiments we have elicited the knee jerk at intervals of 1 per sec., instead of the usual one in 10 sec., but under these conditions also we failed to demonstrate any initial phase of stimulation (Fig. 2).

The latent period of the response is very short. In some experiments the inhibition sets in within 5 sec. of the moment of injection. The knee jerk declines at a rate which varies with the dose. The maximal depression may be reached at once, or develop more slowly over periods of 10–60 sec. The duration of the effect varies widely, but may persist for many minutes, e.g. 5–10 min. or longer. Sometimes no recovery takes place in half an hour or more, or if recovery takes place, it is gradual and incom-

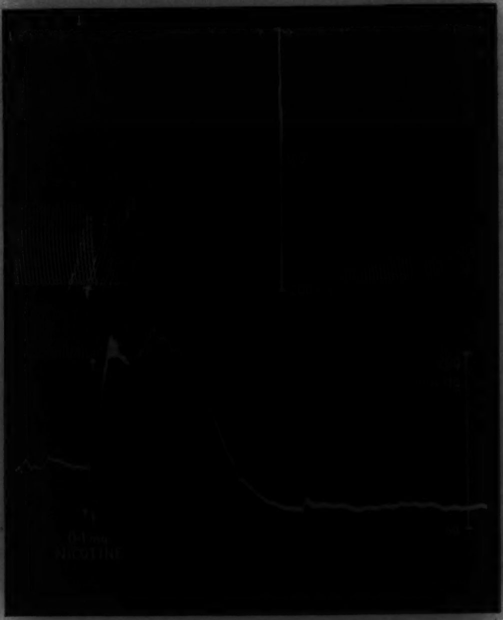

Fig. 1. Cat, chloralose. Records from above downwards are: quantitative record of breathing (Wright's method [1934], inspiration downwards), knee jerk (right side) every 5 sec., contraction of gastrocnemius (left side) stimulated through its motor nerve every 5 sec., carotid blood pressure, time in 30 sec., signal line. Inject 0·1 mg. nicotine into jugular vein.

plete even up to 1 hr. or longer. We have analysed the mechanisms of production of these effects.

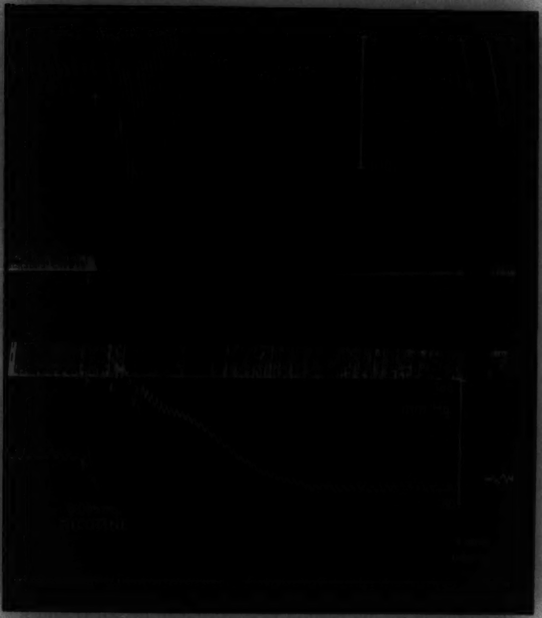

Fig. 2. Cat, chloralose. Records from above downwards are: respiration, knee jerk, nerve muscle preparation and blood pressure as in Fig. 1, knee jerk and gastrocnemius contractions elicited every sec., time in 5 sec. Inject 0·005 mg. nicotine into jugular vein. Pure depression of knee jerk.

Relation to blood pressure

Nicotine produces a rise of blood pressure of varying extent and duration, depending upon the dose used. This rise is not uncommonly preceded by a short initial fall of pressure. The effect on the knee jerk may come on just as rapidly whether the rise of blood pressure is preceded by an initial fall or not. We have previously [Schweitzer & Wright, 1937*a*, *b*] studied the effects of variations in blood pressure on the knee jerk, and have shown that a rise of blood pressure produced by occlusion of the carotid arteries or the injection of tyramine or pituitrin has no effect on the knee jerk. A fall of blood pressure only produces an effect when it is about 100 mm. in extent or when it lowers the pressure to about 40 mm. The effect is then one of stimulation. In the light of these observations it is most improbable that the small initial fall or the greater subsequent rise of pressure is responsible for the knee-jerk changes observed. Furthermore, the recovery of the knee jerk bears no relationship to the course of the blood-pressure curve; the knee jerk may remain absent when the blood pressure has returned to or fallen below its pre-injection level. In some experiments, small doses of nicotine which altered the blood pressure only very slightly produced the usual depression of the knee jerk.

Relation to adrenaline secretion

We have previously shown [Schweitzer & Wright, 1937*b*] that the intravenous injection of adrenaline in doses of 0·2 mg. or upwards may depress or abolish the knee jerk. It is well known that nicotine stimulates the secretion of adrenaline from the adrenal medulla [Cannon *et al.* 1911–12; Gley, 1914], and it was thought possible, therefore, that the adrenaline so liberated might be responsible, in part at least, for the results noted. In a series of animals the adrenal veins were ligated or the glands extirpated. The subsequent injection of nicotine into these animals produced a characteristic depression of the knee jerk.

Relation to respiratory changes

In the doses employed, nicotine produces characteristic respiratory changes, namely a very brief initial apnoea, followed by an intense stimulation (consisting of an increase in rate or depth, or both) and a gradual return to normal. In the decerebrate preparation the stimulation is more persistent and may last for 10 or more minutes. In previous experiments [Schweitzer & Wright, 1937*a*] we have studied the effects of alterations in pulmonary ventilation on the knee jerk. Severe over-ventilation sufficient to produce a considerable reduction in the CO_2 tension of the blood did not affect the knee jerk. The depression in the knee jerk following an injection of nicotine cannot, therefore, be reasonably attributed to the alterations in breathing.

Role of vaso-sensory nerves

In a number of experiments we have denervated the carotid sinuses and divided the vagi prior to the injection of nicotine. The usual changes in the knee jerk were obtained under these circumstances (Fig. 3). These experiments exclude the possibility that nicotine produces its effects reflexly, either *via* the pressor-receptors or the chemo-receptors in the carotid sinus region and the aortic arch. They provide additional evidence that the knee-jerk changes are independent of alterations in the breathing.

Fig. 3. Cat, chloralose. Both carotid sinuses denervated and both vagi cut. Records from above downwards are: respiration, knee jerk, nerve muscle preparation and blood pressure as in Fig. 1, knee jerk and gastrocnemius contractions elicited every 6 sec., time in 5 sec. Inject into jugular vein 1 mg. nicotine.

In the denervated preparation, nicotine produces no stimulation, or only slight stimulation, of breathing [Wright, 1935], but the effect on the knee jerk is still observed.

Action on skeletal muscle

In many of our experiments we have recorded the effects of stimulation of the gastrocnemius through its motor nerve on one side, at the same time as we elicited the knee jerk on the other side. Doses of nicotine that may abolish the knee jerk produce no effect whatever on the response to motor-nerve stimulation, even when applied at a frequency of 1 per sec. G. Briscoe informs us that doses of nicotine of 0·1–1 mg. have no effect on the nerve-muscle responses, not only when low, but also when high rates of stimulation (60 per sec. and over) are employed. In her experiments also, though there was no change in the peripheral response, the knee jerk was abolished. The changes in the knee jerk observed are, therefore, not due to alterations in the response of the quadriceps muscle itself.

We have confirmed this conclusion, however, by the use of the ischaemic preparation previously described (Fig. 4). In many experiments, after the blood supply to the hindlimb had been completely cut off, the intravenous injection of nicotine depressed or abolished the knee jerk. Under the conditions of the experiment the drug could only produce its effects by an action on the central nervous system, or on the peripheral nerves. The second possibility, however, is excluded by the fact that very much larger doses have no action on the nerve-muscle preparation, and therefore cannot be depressing conduction in the motor nerves.

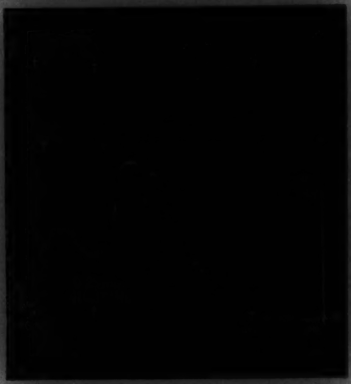

Fig. 4. Cat, chloralose. "Ischaemic preparation." Records from above downwards are: knee jerk (right side) and contraction of gastrocnemius (left side) stimulated through its motor nerve every 5 sec., carotid blood pressure, time in 30 sec., signal line. At "On" clamp abdominal aorta and inferior vena cava. At arrow inject 0·25 mg. nicotine into jugular vein. At "Off" release clamps.

Observations on the decerebrate animal

A certain number of experiments were carried out in decerebrate preparations, which have proved more resistant to the inhibitory action of nicotine than animals under chloralose anaesthesia. This is in no way surprising, as it can be readily supposed that the presence of an anaesthetic facilitates the depressant action of nicotine. In the decerebrate animal (Fig. 5) the depression comes on more gradually, larger doses of the drug have to be employed, and recovery is more rapid and more complete.

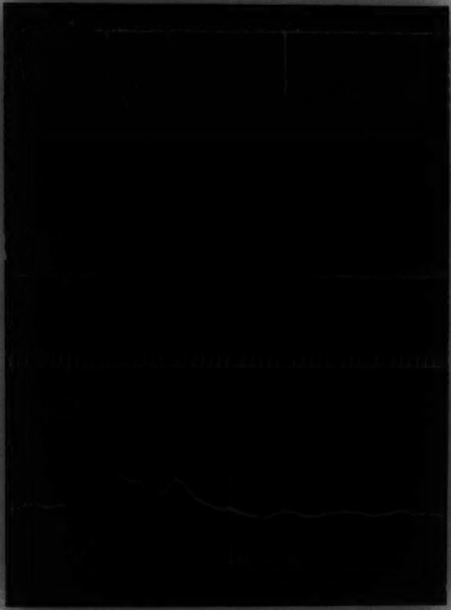

Fig. 5. Decerebrate cat. Records from above downwards are: respiration, knee jerk, nerve muscle preparation and blood pressure as in Fig. 1, knee jerk and gastrocnemius contractions elicited every 1 sec., time in 5 sec.

In none of these animals was stimulation of the knee jerk noted. Special attention was paid to this point in view of Langley's [1901] statement that the excitatory effects of nicotine are depressed by anaesthesia.

In decerebrate animals made spinal by transection of the cord in the lower thoracic regions, the usual effects on the knee jerk were observed. Again, no stimulation was noted.

Anti-strychnine action of nicotine

Contrary to the results of Langley [1918–19], we were able to demonstrate that nicotine exerts an anti-strychnine action. As with all drug antagonisms, the relative doses of the two substances must be carefully adjusted. Our practice has been to inject small doses of strychnine, e.g. 0·1 mg., and repeat the dose until the knee jerk was markedly enhanced, and spontaneous convulsions set in. At this stage large doses of nicotine, e.g. of the order of 2 mg., may abolish both the knee jerk and the convulsions (Fig. 6). The effect, however, is usually short-lived. Recovery takes place much more rapidly than in unstrychninized animals, and the convulsions may subsequently return.

Fig. 6. Cat, chloralose. Knee jerk (right side), contractions of gastrocnemius (left side) stimulated through its motor nerve every 5 sec., carotid blood pressure, time in 5 sec., signal. 0·1 mg. strychnine was previously injected four times in succession into the jugular vein, leading to an increase in the knee jerk and onset of convulsions. At arrow: inject 2 mg. nicotine into the jugular vein. Convulsions reappear and knee jerk temporarily abolished.

DISCUSSION

Our experiments show that nicotine exerts a depressant action on spinal reflexes in the cat. In view of the contradictory statements in the older literature and the known initial excitatory action of nicotine on autonomic ganglia, we paid special attention to possible excitatory effects of the drug on the central nervous system. We were unable, however, using the patellar reflex as a typical spinal reflex, with the dose of nicotine employed (1μg.–2 mg./kg. body weight) to demonstrate any excitatory effect of the drug on the knee jerk, nor did we notice any other signs of heightened central excitation, such as convulsions. Even very transient excitatory changes in the spinal reflexes occurring within at least 1 sec. of injection would have been recorded in the series of experiments in which the knee jerk was elicited at intervals of 1 sec. The possibility that any excitatory effect of nicotine on the central nervous system was suppressed by the anaesthetic used was tested in experiments on decerebrate preparations, in some of which the spinal cord was subsequently divided in the lower thoracic region. No sign of central excitation was observed in any of these experiments. It is not surprising that decerebrate cats showed a greater resistance to the depressant effect of the drug than animals under chloralose anaesthesia, as in the former preparation there is increased muscular tone due to a greater discharge from the anterior horn cells.

Further evidence for the central depressant action of nicotine was obtained by the experiments in which nicotine was pitted against heightened excitability of the spinal cord produced by a preceding injection of strychnine. As already pointed out, drug antagonisms can only be successfully demonstrated when suitable relative doses are employed. In our experience, nicotine depresses or even abolishes the increased patellar reflex and the convulsions induced by intravenous injections of strychnine.

The role of the carotid sinus and aortic nerves was carefully studied. It is known that increased pressure within the carotid sinuses may inhibit the knee jerk and decrease muscular tone in dogs (full discussion, cf. Schweitzer, 1937). Furthermore, central vagus stimulation in the cat decreases the patellar reflex and may inhibit strychnine convulsions [Schweitzer & Wright, 1937*a*, *e*; Schweitzer, 1937]. The increase of arterial blood pressure following an intravenous injection of nicotine might, therefore, be responsible for the phenomena observed. Our experiments on animals in which the pressor-receptor and chemo-receptor mechanisms of the carotid sinus region and the aortic arch were eliminated show that the depression or abolition of the knee jerk by nicotine is not exclusively brought about reflexly *via* the vaso-sensory nerves.

We conclude that nicotine depresses spinal reflexes and counteracts the heightened reflex activity and the convulsions induced by strychnine by a direct action on the spinal cord. This conclusion is partly supported by recent work of Therman [1938] and Barron & Matthews [1938]. Therman reported a paralyzing effect of nicotine on the retinal action potentials of the excised frog's eye, consisting mainly of a decrease and slowing down of the reactions. Barron & Matthews observed that nicotine abolished in frogs the slow dorsal root potentials which probably originate in the grey matter of the spinal cord. de Kleyn [1938] studied the effects of acetylcholine and nicotine on vestibular responses. If a solution of either drug is applied to the orbital contents in which the ocular muscles had been dissected free, a distinct retardation of the frequency of the nystagmus occurs, not only on the affected side, but also in the opposite eye. In view of the fact that intravenous injection of the drugs did not influence the nystagmus, de Kleyn concluded that the drug could not have acted on the central nervous system. It is difficult, however, to see how any peripheral action could have produced the effects noted, especially in the other eye, and it is more probable that the drugs were absorbed by some non-vascular route into the central nervous system.

SUMMARY AND CONCLUSIONS

1. Nicotine depresses or abolishes the knee jerk in cats under chloralose anaesthesia, and in the decerebrate or spinal preparation. It also depresses or abolishes strychnine convulsions. Excitatory effects have not been observed, even with small doses (0·005 mg.).

2. This action is independent of changes in respiration and blood pressure. It is still obtained after denervation of both carotid sinuses and section of both vagi, and exclusion of both adrenal glands.

3. The depressant effect of nicotine on the knee jerk is not due to changes in the response of the peripheral muscles to motor nerve stimulation. It is obtained in "ischaemic preparations" in which the drug does not reach the hindlimbs.

4. It is concluded that nicotine inhibits spinal reflexes by a direct action on the spinal cord.

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Note added in proof. Experiments reported by B. **Libet** & R. **Gerard**, 1938, *Proc. Soc. exp. Biol., N.Y.*, **38**, 886 (which appeared while this paper was passing through the Press), provide independent testimony in support of the central depressant action of nicotine in the frog and cat. In "ischaemic" experiments in cats intravenous injection of nicotine abolished crossed and uncrossed spinal reflexes. Local application of 2·5 mg./kg. in the cat blocked tactile action potentials in the thalamus.

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THE ABSORPTION AND EXCRETION OF IRON FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION

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FROM a study of the literature and from experimental work on patients and on normal persons McCance & Widdowson [1937] have suggested that the capacity of the body to excrete iron may be very much less than has generally been supposed. It may, in fact, be negligible. There is no dispute over urinary iron, which is agreed by all to be very small and almost constant in amount. The "new" theory differs from those currently accepted in that it postulates no regulatory excretion of iron by the intestine. Admittedly, traces must be derived from abraded cells and glandular secretions, but essentially the iron found in the faeces represents unabsorbed iron. The present paper records the results of further experiments planned to study whether or not the human intestine can excrete iron and by so doing regulate the amount of iron in the body. The investigation has been carried out on normal human adults and has consisted of three stages. In all a mixed diet with a fairly low iron content has been taken. In stage 1 the food constituted the sole source of iron. The subjects, as expected, were found to be in balance, and this was an essential preliminary finding. Current theories of iron metabolism would explain this by stating that measurable quantities of iron were being absorbed every day and that the subjects were maintained in iron equilibrium by a process of regulated or controlled excretion. If this were true, the fact that they were in balance showed that they were saturated with iron up to normal capacity. Consequently any increase in the quantity of iron in the body should have been followed by the excretion of a corresponding amount. The new theory would explain the facts by postulating that practically no iron was being absorbed or excreted as the metal passed through the intestinal tract. In stage 2 balance experiments were carried out with the intakes of iron raised 50–100 % by medicinal supplements. The object of this stage was to test the response of the subjects to a "physiological" increase of iron taken by mouth. Should they continue in balance, it was clear beforehand that either theory would be able to cover the results. Positive balances would be difficult to explain on current theories but easy to explain on the new theory (*vide infra*). In stage 3 balance experiments were carried out as before and iron was injected intravenously in amounts similar to those administered as medicinal supplements in stage 2. According to current theories iron so given to subjects in iron equilibrium should certainly have led to the excretion of the administered iron in the faeces. According to the new theory, however, the injected iron should have been retained.

EXPERIMENTAL ORGANIZATION

The subjects consisted of three women and three men. E. M. W., M. M. and M. H. L. were women aged respectively 30, 22 and 24. All were research workers in the department. They menstruated regularly and the experimental stages were arranged to fall into three successive intermenstrual periods. R. M. L., R. H. E. and R. A. M. were men aged 23, 23 and 39 respectively. The first two were medical students, the last a physician. All the subjects were in good health when the experiment began and remained so throughout.

Each stage consisted of 14 experimental days, of 2 or 3 preliminary days and of 1 or 2 concluding days. During the preliminary and concluding days the experimental diet was eaten but the food was not weighed. The experimental days were divided into two periods each of 7 days. The technique was almost exactly the same as that given by Widdowson & McCance [1937] for stages 1 and 3 of the investigation then being described. In this experiment, however, (1) the aliquot of wet faeces taken to be ashed was not always 30 g. but varied between 30 and 50 g., (2) after the ashed faeces had been treated with concentrated HCl the solution was merely taken to dryness on the sand bath and not heated for some hours. The iron administered by mouth during stage 2 was a ferric ammonium citrate preparation and was made up in solution with salts of magnesium, potassium and zinc, since a study of the way in which the body dealt with these metals was being carried out at the same time. The solution was measured out daily with a marked pipette and aliquots taken with the same pipette for analysis. The iron administered intravenously during stage 3 was also an ammonium citrate preparation obtained as a solution in ampoules from British Drug Houses, Ltd., and labelled "Injection of iron, B.P." It was administered in combination with calcium, magnesium and zinc in the following way. A sterile 20 c.c. syringe, fitted with a two-way attachment, was connected (*a*) to a reservoir of sterile saline, (*b*) to a piece of rubber tubing about 40 cm. in length. To the distal end of this the needle was connected by a piece of stout rubber tubing about 5 cm. long and a short piece of glass tubing. The whole system was filled with saline before the needle was inserted. When the needle was in the vein, the rubber tubing between the needle and the glass tubing was clamped off with a pair of Spencer Wells forceps about 4 cm. from the needle, and through this piece of rubber tubing four fine needles were inserted each connected directly to a syringe. Two of these syringes contained respectively calcium and magnesium gluconates, the third contained a solution of zinc chloride and the fourth the iron preparation of which 2 c.c. (=7 mg. of Fe) were administered daily. The contents of the four syringes were slowly injected, the whole process taking 30–45 min. With a little practice the subjects managed to do this for themselves. The plungers of the syringes were pressed gently and slowly in rotation, the pace being so regulated that all four syringes were emptied at about the same time. After the syringes had been emptied the clip on the tubing was removed and the injection material still in the tubing swept into the vein by a stream of saline. The investigations which have been made upon potassium, calcium, magnesium and zinc will form the basis of a separate paper.

RESULTS AND DISCUSSION

The balances of all six subjects during stage 1 are shown in Table I. In the 14 experimental days the iron intakes of the six subjects varied from 82·2 to 120·3 mg. (5·9–8·6 mg./day). These variations were due to appetite and individual food selection. The distribution of iron between the urine and faeces is characteristic of the way in which iron is excreted and requires no

TABLE I. Iron balances on low iron intakes by mouth

Subject	Intake mg./14 days	Excretion (mg./14 days)			Balance
		Urine	Faeces	Total	
E. M. W.	93·3	1·6	86·9	88·5	+ 4·8
M. M.	87·0	1·1	94·0	95·1	− 8·1
M. H. L.	82·2	1·0	79·0	80·0	+ 2·2
R. M. L.	102·0	1·1	97·6	98·7	+ 3·3
R. H. E.	118·5	1·6	132·0	133·6	−15·1
R. A. M.	120·3	1·4	117·5	118·9	+ 1·4
Totals	603·3	7·8	607·0	614·8	−11·5

comment. On these intakes four subjects were in positive balance and two in negative balance. R.H.E., who showed the largest negative balance, was in iron equilibrium during the second 7 day period, and it is possible that his negative balance was due to an iron contamination or to an error in marking the faeces during the first 7 days. On the whole, however, the balances are close and the group results of −11·5 mg. on a total intake of 603·3 mg. is as near a balance as the experimental difficulties of this sort of work are likely to allow. These six persons therefore were concluded to be "in balance" on these intakes of iron and to be suitable for the investigation.

The balances of all the subjects during stage 2 are shown in Table II. It will be recalled that the object of this stage was to test the response of persons in iron equilibrium to a moderate increase of iron taken by mouth. Accordingly, each person's intake was raised by 82·8 mg. in the 14 days. The food intakes were also slightly larger so that the total intakes ranged from 167·4 to 225 mg. in the fortnight (12–16 mg./day). On these intakes the output in the urine was not significantly changed. The output in the faeces rose with the intake by mouth and four subjects were found to be in positive and two in negative balance. One of the positive balances was relatively large and weighted the final result, but a group balance of +27·6 mg. on a total intake of 1160 mg. is probably within experimental error. It is evident therefore that these persons were sensitive to physiological variations of intake and responded by raising their rates of excretion to approximately the same extent. Current theories would explain this by laying the burden of the regulation on the mucosa of the intestinal tract, but the new theory by stating that only minute amounts of iron were absorbed either on the low or the raised intake.

TABLE II. Iron balances on higher iron intakes

	Intake mg./14 days		Total intake mg./14days	Excretion mg./14 days		Total excretion mg./14days	
Subject	Food	Medicine		Urine	Faeces		Balance
E. M. W.	94·4	82·8	177·2	2·3	167·2	169·5	+ 7·7
M. M.	99·2	82·8	182·0	0·9	185·0	185·9	− 3·9
M. H. L.	84·6	82·8	167·4	1·2	158·5	159·7	+ 7·7
R. M. L.	112·5	82·8	195·3	1·7	205·1	206·8	−11·5
R. H. E.	130·6	82·8	213·4	1·1	205·5	206·6	+ 6·8
R. A. M.	142·2	82·8	225·0	1·7	202·5	204·2	+20·8
Totals	663·5	496·8	1160·3	8·9	1123·8	1132·7	+27·6

It is known that if the intake of iron by mouth is sufficiently high, people tend to go into positive balance [Brock & Hunter, 1937; Fowler & Barer, 1937; Hutchison, 1937]. This would hitherto have been explained by supposing that excretion was not able to keep pace with absorption, but Widdowson & McCance [1937] have shown that the absorbed iron is not excreted after the intake is restored to normal. The new theory supposes that the amount of iron in the body is regulated by the amount absorbed and this in turn is regulated among other things by the amount taken by mouth. Hence positive balances on high intakes are simply explained. The small positive balance of the group of six subjects in stage 2 may be an indication of positive balances due to raising the intake, but it is impossible to say whether this is so or not.

Table III. Iron balances during the intravenous administration of iron

Subject	Intake (mg./14 days)		Excretion (mg./14 days)			Balance including injected iron	Balance neglecting injected iron
	Food	Intravenous	Urine	Faeces	Total		
E. M. W.	115·6	98	3·0	111·0	114·0	+ 99·6	+1·6
M. M.	106·2	98	2·3	104·3	106·6	+ 97·6	−0·4
M. H. L.	107·2	98	2·8	107·5	110·3	+ 94·9	−3·1
R. M. L.	160·5	98	3·1	166·0	169·1	+ 89·4	−8·6
R. H. E.	164·5	98	2·0	160·0	162·0	+100·5	+2·5
R. A. M.	151·6	98	3·4	153·8	157·2	+ 92·4	−5·6
Totals	805·6	588	16·6	802·6	819·2	+574·4	−13·6
Correcting for the extra excretion in the urine						+583·2	−4·8

The results of administering the supplementary iron by intravenous injection are shown in Table III. The amount of iron in the food was again a little higher and ranged from 106·2 to 164·5 mg. in the 14 days (7·6–11·7 mg./day). In addition to this, 98 mg. in all were administered to each subject intravenously (7 mg./day). Turning to the output it will be noted that the urinary iron was higher in each subject during this stage and that the total quantity in the urine was about twice what it had been in stages 1 and 2. This is not surprising, since it has been known for a long time [Jacobi, 1891; Henriques & Roland, 1928] that if iron is administered intravenously, some of it tends to find its way into the urine. The amount of iron so excreted, however, in this experiment was about 1·4 p.c. of the amount injected and it is almost certainly of no practical importance, since no extra iron is excreted by this route when large doses of iron are taken by mouth and large quantities of iron are being absorbed. This excretion therefore probably takes place while the injection is actually being made and before the injected iron has been removed from the plasma. The amount of iron in the faeces was in all subjects close to the amount in the food. This discovery was the culminating point of the whole experiment. Actually, if the injected iron is neglected altogether, two of the subjects were in slight positive balance and four in slight negative balance. The group balance of −13·6 mg. on an intake by mouth of 806 mg. is well within experimental error, and even this negative balance is subject to a correction for the extra iron found in the urine. Making this correction by using the figure for urinary iron found in stage 1 the group balance becomes −4·8 mg. It may be concluded, therefore, that *none* of the injected iron was excreted by the gastro-intestinal tract. Since the subjects were known to be in iron equilibrium beforehand and fully saturated with iron up to normal capacity, the injected iron should have been excreted into the gut in reasonably large amounts if the gut plays any real part in regulating the amount of iron in the body. The present results are entirely in agreement with the new theory according to which the capacity of the body to excrete iron is strictly limited and probably negligible.

IMPLICATIONS AND CONCLUSION

These experiments raise a number of interesting points. In the first place it is curious that the three normal women were not in positive iron balance in their intermenstrual periods. Assuming these women to lose 20 mg. of iron/month [Ohlson & Daum, 1935; Barer & Fowler, 1936] they might be expected to absorb 10 mg./fortnight to make good this loss, i.e. 60 mg. between them in stages 1 and 2. In these two stages, however, their joint positive balance was only 10·4 mg. It would be interesting to know at what time during the menstrual cycle these women absorbed the iron necessary to make good their loss. In the second place, now that it has been shown that the intestine does not excrete iron, is it wise to adhere without question to the belief that it regulates by excretion the amount of calcium or magnesium in the body, or indeed the amounts of any of the physiological or non-physiological metals? It would be premature at the moment to go into this interesting possibility, but the matter is being investigated. Should it be proved that the gut does not excrete these metals, work on the metabolism of the divalent metals will be immensely facilitated.

SUMMARY

1. In the first stage of this experiment three men and three women were placed upon diets containing 5·9–8·6 mg. of iron/day and shown to be in balance.

2. In the second stage the oral intakes were raised to 12–16 mg. of iron/day and the subjects were again shown to be in balance, i.e. all the supplementary iron was excreted.

3. In the third stage 7 mg. of iron were injected intravenously every day into the same subjects, whose intakes by mouth now ranged from 7·6 to 11·7 mg./day. Within the limits of experimental error none of the injected iron was excreted into the gastro-intestinal tract. About 1·4 p.c. of it was excreted in the urine, but reasons are given for believing that this is of no practical significance in iron metabolism.

4. These results are considered to confirm the theory that the intestine has no power of regulating by excretion the amount of iron in the body.

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LIBERATION OF ACETYLCHOLINE BY THE PERFUSED SUPERIOR CERVICAL GANGLION

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In earlier experiments from this laboratory on the perfused ganglion preparation, stimulation of the preganglionic nerve regularly caused the appearance of acetylcholine (ACh.) in the venous effluent [Feldberg & Gaddum, 1934], while stimulation of the vagus, of which the nodose ganglion was included in the perfusion, or of the sympathetic post-ganglionic branches, had no such effect [Feldberg & Vartiainen, 1934]. This and other evidence led to the definite suggestion that acetylcholine is liberated at the synapses by the arrival of preganglionic impulses, and plays an essential part in the synaptic transmission of the excitatory process, a view which much later evidence has further supported. Recently Lorente de Nó [1938], with the object of testing other possibilities as to the site of liberation of acetylcholine in the ganglion, has repeated the earlier experiments, using essentially the same methods, with modifications of detail. In a proportion of these experiments, especially when the methods used by Feldberg *et al.* were closely followed, Lorente de Nó obtained similar results. In other experiments his results were irregular, and, being impressed by the possibility of damage to the ganglion during its preparation and perfusion, he elaborated precautions to protect it from injury. These ultimately involved a very prolonged dissection in a moist room at 34° C., and perfusion, in some cases at least, at low pressures. Under these conditions, although the highest outputs of acetylcholine were still usually seen with preganglionic stimulation, it frequently appeared at other times, and sometimes failed to appear in response to preganglionic impulses. Lorente de Nó concluded that ACh. may be liberated in the ganglion when no impulses are arriving at the synapses, that its liberation is favoured by injury to the ganglion, and that its "metabolism is not a process which is specific to

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synaptic junctions". The experimental findings on which he based this conclusion may be detailed as follows:

(1) The liberation of significant amounts of ACh. in the absence of stimulation.

(2) Liberation of ACh. by stimulation of the postganglionic sympathetic fibres, or of the vagus, as well as by stimulation of preganglionic fibres.

(3) Delayed output of ACh., outlasting the stimulation by many minutes.

(4) Failure of preganglionic stimulation to discharge ACh. from ganglia which had been prepared with special precautions to avoid injury.

(5) Histological evidence of severe damage in those ganglia which had liberated ACh. on stimulation.

At the time of the publication of Lorente de Nó's paper I had for some time been engaged with experiments on the perfused ganglion, using an improved method of perfusion devised in co-operation with Dr G. L. Brown, in which the perfusion fluid was diluted blood instead of simple Locke's solution. Confirmation of the fundamental points of the earlier experiments had not been the direct object, but I had had the opportunity, in every experiment, of observing the close correspondence between the appearance of ACh. in the venous fluid, and the effective stimulation of the preganglionic nerve. With the improved perfusion method the ganglion was regularly maintained in good condition, without perceptible oedema, for several hours, during which repeated short periods of preganglionic stimulation had been observed to cause practically identical outputs of ACh. There was, accordingly, a favourable opportunity of repeating the original experiments under better conditions, and with special attention to the points at which Lorente de Nó had obtained results conflicting with those of the earlier workers. It will be seen that the results of my experiments are in close agreement with those of Feldberg & Gaddum and Feldberg & Vartiainen, and that I have failed to reproduce the irregularities observed by Lorente de Nó, even when I have varied the conditions with the object of favouring their appearance.

METHODS

Cats under chloralose anaesthesia were used in all the experiments. The dissection prior to perfusion was as described by previous workers [Feldberg & Gaddum, 1934; Feldberg & Vartiainen, 1934]. The vagus nerve and accompanying structures, with the exception of the sympathetic postganglionic trunk, were tied between the ganglion and the skull, but not cut. During the dissection the wound was frequently bathed with warm saline, and great care was taken to avoid any mechanical injury to the ganglion. Elaborate precautions to prevent cooling and drying were not taken, and the temperature of the ganglion doubtless fell considerably during the operation, which usually required 30–40 min. for its completion. As soon as the dissection was finished, heparin was injected into the general circulation, in a dose sufficient to keep the blood incoagulable for the whole period of the experiment. The animal was then usually left, with the ganglion protected by closure of the wound and still retaining its normal blood supply, for an hour or more before the actual perfusion was begun.

In most of the experiments the perfusion fluid was a tenfold dilution of the cat's own heparinized blood in normal Locke's solution, with the addition of 1 : 250,000 eserine sulphate. The blood was diluted immediately it was drawn, and then passed twice through a No. 3 Jena sintered-glass filter. A small Dale-Schuster pump maintained a slightly pulsatile perfusion pressure of 100–150 mm. Hg; at these pressures the perfusion rate through the ganglion preparation was from 0·5 to 1·0 c.c. per min. A glass-wool filter was interposed between the pump and the arterial cannula. The perfusion fluid was warmed to a constant temperature of 38–39° C. by means of a coil of resistance wire built into the cannula, which was of the type devised by Gaddum and described by Feldberg & Vartiainen [1934], but shorter, and with the heating coil extending almost to the nozzle inserted into the artery, the fluid, containing blood, being thus at no point heated above 40° C.

The temperature in the neighbourhood of the ganglion was not lower than 35° C. during the course of the perfusion. In a few experiments, which will be separately considered, the fluid used was plain Locke's solution containing 1 : 250,000 eserine sulphate, perfused either by means of the pump, or by static pressure from a raised reservoir. The nerves to be stimulated were placed, at the beginning of the experiment, on glass-shielded platinum or silver electrodes, or in fluid electrodes of the Collison pattern, and left without disturbance until the experiment was completed. The stimuli were in all cases condenser discharges at a frequency of 10 per sec. A continuous record of the response of the nictitating membrane provided a check on the effectiveness of pre- or postganglionic stimulation.

The perfusate was collected in ice-cooled vessels, and either tested immediately on the leech preparation, or kept in ice until the assay could be made. Control experiments showed that there was no appreciable destruction of ACh. by cat's blood at the dilution employed, and with the amount of eserine added, when kept at 0° C. during a period of several hours. The leech strips used always responded well to ACh. in 2×10^{-9} dilution, and usually to considerably lower concentrations.

EXPERIMENTAL

Failure of acetylcholine to appear in the perfusion fluid in the absence of stimulation

The presence of blood in the perfusion fluid, when this is first applied to the test preparation, sometimes provokes a small contraction, even when no ACh. is present. This non-specific response disappears on repeated application of the diluted blood, and in any case usually differs in form from that produced by solutions of ACh. In a few experiments (four out of fourteen), however, the first samples of fluid, collected at the beginning of the perfusion, showed an activity which could not be explained in this way, and which was doubtless due to the presence of ACh. The concentration of ACh. in such control samples, however, never exceeded 1×10^{-9}, or about 5 % of the concentration later found when the preganglionic nerve was stimulated. Later samples invariably failed to show even this slight activity. This small, temporary, and inconstant "spontaneous" output of ACh. is probably not due to injury discharge of impulses in the cut preganglionic trunk, since this was always divided about 2 hr. before. On the other hand, Lorente de Nó's suggestion, that this small initial output, when it occurs, is due to damage of the ganglion cells, has no real evidential basis. Brown & Feldberg's [1936*a*] evidence indicates that the ACh. extractable from the ganglion is located at the preganglionic nerve endings and dependent on their integrity; so that, if the ganglion were so injured in preparation as to liberate ACh., it would be natural to suppose that it would be liberated by the injury, as by nerve impulses, from the preganglionic nerve endings and not from the ganglion cells. On the other hand, after a dissection conducted so as to avoid directly touching the ganglion, and a long subsequent interval without eserine before the perfusion is begun, it seems little likely that even the traces of ACh. sometimes found in the early effluent could have been liberated by injury to the ganglion during dissection. While, however, the sympathetic ganglion itself is carefully avoided in dissection, a number of nerves and branches are necessarily cut in isolating the small mass of tissue, including both vagus and sympathetic ganglia, which is subsequently perfused. From the cut ends of these nerves, retracted into the perfused tissues, it is likely that a small leakage of ACh. may continue for some time, and contaminate the early runnings of the eserinized perfusion fluid. In any case, this initial "spontaneous" output has, in my experiments, been so small and so evanescent as never to complicate the clear effect of preganglionic stimulation.

Release of acetylcholine by preganglionic stimulation

Stimulation of the preganglionic trunk never failed, in thirteen experiments, to cause the liberation of considerable quantities of ACh. (Fig. 1, Table I). The effect could be repeated, with short periods of stimulation, an indefinite number of times. Calculation of the quantity liberated per single maximal shock gave values lying remarkably close together. Thus in twenty-four out of twenty-six stimulations, the output per shock was between 0·000015 and 0·000035 μg., with a mean of 0·000024 μg.; in the other two cases the outputs per shock were 0·000008 and 0·000053 μg. respectively. These values are somewhat lower than those obtained by Feldberg & Vartiainen [1934], 0·000066 and 0·0001 μg., in two experiments made especially for the purpose of such estimates; I have also found values in the latter range, in experiments in which the perfusion fluid was eserinized Locke's solution rather than diluted blood. In any one of the experiments with diluted blood as perfusion fluid, the output per shock either remained nearly constant at all stages of the perfusion, or showed a slight falling off in the later stimulation periods. The variation, from one animal to another, of the quantity of ACh. liberated by each maximal stimulus, is scarcely greater, however, than the variation observed in the ACh. content of individual ganglia, and lends no support to the suggestion that the effectiveness of stimulation in liberating ACh. is proportional to the degree of injury suffered by the ganglion.

The transient relaxation of the contracted nictitating membrane during the initial period of a continuous, rapid, preganglionic stimulation, shown by Brown & Feldberg [1936c] to be due to the accumulation within the ganglion of an excess of free ACh., has been regularly observed in my experiments, when such a long-continued stimulation was applied.

Failure of vagus and postganglionic stimulation to liberate acetylcholine

Maximal stimulation of the vagus peripheral to the ganglion (in thirteen experiments), or of the postganglionic sympathetic nerves (in six experiments), failed without exception to cause the liberation of detectable quantities of ACh. (Fig. 1, Table I). Preganglionic stimulation

Fig. 1. Perfusion with diluted blood. Eserine 1 : 250,000. Perfusion rate 0·6 c.c. per min. Leech assay of successive 10 min. samples. *Pre.* = preganglionic stimulation, 9 min.; rest 1 min. *C* = control (no stimulation). *V* = vagus stimulation, 10 min. *Post.* = postganglionic stimulation, 10 min. Leech preparation sensitive to ACh. 5×10^{-10}. Change to clean Ringer solution at ×, with drum stopped.

TABLE I

	ACh. liberated (μg.)				
Exp.	1st control sample	Later control samples	Preganglionic stimulation	Postganglionic stimulation	Vagus stimulation
1	None	None	0·100 (8)	None (5)	None (8)
			0·083 (8)		None (8)
2	None	None	0·036 (8)	None (8)	None (8)
			0·078 (8)		
			0·084 (8)		
3	None	None	0·088 (8)	None (10)	None (10)
			0·092 (8)		
4	None	None	0·161 (8)	None (8)	None (8)
			0·115 (8)		None (8)
			0·110 (8)		
5	0·004	None	0·280 (8)	None (10)	None (8)
			0·170 (8)		None (10)
6	0·005	None	0·022 (1·5)	—	None (5)
			0·017 (1·5)		
7	None	None	0·027 (1·5)	—	None (5)
8	None	None	0·032 (3)	—	None (5)
9	0·003	None	0·085 (5)	—	None (5)
10	None	None	0·044 (4)	—	None (5)
11	None	None	0·040 (4)	—	None (5)
12	None	None	0·060 (4)	None (10)	None (8)
			0·051 (4)		None (10)
			0·096 (9)		
			0·100 (9)		
13	None	None	—	—	None (30)
14	0·002	None	0·050 (4)	—	—
			0·060 (4)		
			0·060 (4)		
			0·050 (4)		
			0·040 (4)		
			0·035 (4)		

The table summarizes the results of all the experiments in which the perfusion fluid was diluted blood. Figures in parentheses indicate the duration of stimulation in each case; the stimulation frequency was always 10 per sec. Values for ACh. outputs during control periods, where obtained, refer to 10 min. periods.

liberated ACh. in the usual amount in all these experiments, except No. 13, in which the cervical sympathetic nerve had been cut 3 days previously. In this case stimulation of this nerve caused no transmission of impulses through the sympathetic ganglion and no output of ACh. [see MacIntosh, 1938; Bacq & Coppée, 1938].

Absence of "delayed output" of acetylcholine

In most of the experiments the sample of perfusate corresponding to each stimulation period was collected during the progress of stimulation and a further 1 or 2 min. after its end. The succeeding sample was then completely inactive, or showed at the most a barely detectable trace of activity. In certain special experiments an attempt was made to determine more exactly how close a correspondence obtained between the period of preganglionic stimulation and the period during which ACh. was present in the perfusate. For this purpose the venous effluent was collected in 30 sec. samples, which were separately assayed, the perfusion pressure being raised to 180 mm. Hg, in order to permit the collection of a sufficient volume of fluid in each period of this brief duration, and to reduce the effect of the dead space in the venous cannula, which had a volume of 0·08 c.c., and the collecting vein. The result of such an experiment is shown in Fig. 2. It will be seen that ACh. in detectable concentration is already present in the first half-minute sample collected during preganglionic stimulation; the output increases in succeeding periods, falls immediately in the 30 sec. following the end of stimulation, and in the next 30 sec. has returned to zero. With slower rates of perfusion,

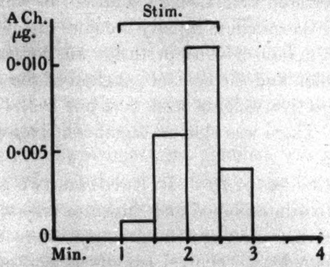

Fig. 2. Perfusion with diluted blood, eserine 1 : 250,000. Perfusion rate 1·2 c.c. per min. Rectangles indicate ACh. output in successive ½ min. periods. *Stim.* = preganglionic stimulation for 1½ min.

traces of ACh. could occasionally be detected in samples collected during the second minute after the end of stimulation, but not in later samples.

The diffusion of liberated ACh. into the vessels of the ganglion, and its removal from these and from the outflow cannula, is thus complete within a period of from 30 sec. to 2 min., under different conditions. As Lorente de Nó has pointed out, it is obviously impossible to show, by such a method as this, whether or not there is *any* production of ACh. which outlasts the transmission of impulses through the ganglion. The present results, however, are certainly compatible with the opinion that there is not.

Absence of evidence of injury in the perfused ganglion

No sign of oedema could be detected by simple inspection, in any preparation perfused with dilute blood, even after perfusion had continued for several hours; in this respect there was a very marked contrast to preparations perfused with Locke's solution, in which the connective tissue surrounding the ganglion rapidly becomes distended with fluid. In three experiments, in each of which perfusion was continued for over 2 hr., the perfused ganglion and the control ganglion of the opposite side were weighed: the respective weights were 10·2 and 9·4, 11·9 and 12·3, and 12·1 and 11·3 mg. There was thus no significant increase in weight as a result of perfusion.

Through the kindness of Dr J. R. Perdrau, two ganglia, which had been perfused with diluted blood and shown to release ACh. as usual on preganglionic, but not on vagus stimulation, were examined histologically, and compared with control ganglia of the opposite side, both being excised after fixation with the minimum of dissection. Fixation was by Bodian's [1936] method, used by Lorente de Nó, which involves a short preliminary perfusion even of the control ganglion *in situ* with saline, and then of both with diluted alcohol. Sections were stained by Bodian's silver method, or with haemotoxylin-eosin, toluidine blue, or Weigart's fibrin stain. All sections showed considerable shrinkage of ganglion cells, to be attributed to the use of alcohol fixation; but in this and in all other respects there was no recognizable difference between the perfused and stimulated and the control ganglia. In particular, the perfused specimens showed little or no oedema of either the ganglion or its supporting tissue; mild leucocytic infiltration, involving the extracapsular tissue only, was observed in one of the perfused preparations, but also in one of the controls; blocked capillaries and fibrin were absent.

Perfusion with Locke's solution

In an attempt to find the reason for the discrepancy between the results of Lorente de Nó and those obtained in this laboratory, a number of perfusions were carried out with plain, eserinized Locke's solution. The effects of various perfusion pressures, ranging from 35 to 200 mm. Hg, and of pulsatile pressure (Dale-Schuster pump) as compared with static pressure (raised reservoir), were tested. These experiments, however, with one exception, gave results quite in accord with those obtained when the perfusion fluid was diluted blood. Preganglionic stimulation regularly caused the appearance of ACh. in the perfusate; vagus and postganglionic stimulation had no such effect. As might be expected, the removal of liberated ACh. by the perfusion fluid was somewhat slower at the lowest perfusion pressures: thus traces of activity, not exceeding 5% of that present in "stimulation" samples, were under such conditions still detectable in samples of effluent collected in a period beginning 2 min. after the end of stimulation. A little ACh., not exceeding 2×10^{-9} in concentration, was sometimes present in the first control samples collected after the beginning of perfusion, but not in later samples prior to preganglionic stimulation.

In the one aberrant experiment the ganglion was perfused for 2 hr. with eserinized Locke's solution at the high, pulsatile pressure of about 200 mm. Hg. It became very oedematous, but still responded to preganglionic stimulation with a good output (0·00005 μg. per shock) of ACh. The pump was then disconnected, and perfusion continued from the reservoir at a static pressure of 40 mm. Hg, the perfusion rate falling to 15% of its previous value. Preganglionic stimulation now failed to cause the appearance of any detectable ACh. in the perfusate, although there was a good response of the nictitating membrane; and further stimulation was equally ineffective in bringing out ACh., even after the original high, pulsatile pressure had been restored. This experiment is quoted, as being the single one in which, even under conditions made deliberately unfavourable, effective preganglionic stimulation was not accompanied by demonstrable release of ACh. from the ganglion. It is to be noted that, after such deliberate distension of the tissues by high pressure, the departure from the normal result, with subsequent slow perfusion, was not the appearance of ACh. without stimulation, but its failure to reach the effluent when the preganglionic nerve was stimulated. In another experiment, in which exactly the same sequence was applied, preganglionic stimulation caused the normal appearance of ACh. in the effluent at all stages of the perfusion. The most probable explanation of this one case, in which no discharge of ACh. could be detected, is the occurrence, with the change from high pulsatile to low static pressure, of a localized obstruction, causing the perfusion thereafter to pass chiefly through the tissues outside the sympathetic ganglion. Earlier experience in this laboratory had shown the necessity of great care with regard to filtration of the perfusion fluid, particles of microscopic size being liable, by blocking small arteries, to produce retardation and irregular distribution of the flow through the perfused tissue. It is not difficult to imagine that an abrupt and wide variation of the perfusion pressure might cause the flow thus to be largely diverted from the ganglion itself.

Acetylcholine in the venous blood of a stimulated ganglion

Feldberg & Vartiainen [1934] observed that preganglionic stimulation in the eserinized cat may lead to the appearance of ACh. in the venous blood coming from the ganglion. I have made a few similar experiments, but in this case have obtained varying results. The ganglion was isolated as for perfusion, but retained its natural blood supply from the common carotid; atropine and massive doses of eserine (2–6 mg. per kg.) were given intravenously, and the animal's blood was made incoagulable with adequate doses of heparin. The blood flow through the preparation was always extremely slow (1 c.c. in 12–40 min.), and samples usually showed slight activity in the absence of stimulation. In three out of five experiments, preganglionic stimulation failed to increase the ACh. concentration of the venous blood, although it could be shown that the blood contained sufficient eserine to preserve ACh. In the other two experiments, preganglionic stimulation regularly produced a clear-cut augmentation of the ACh. output; in one of these the vagus was stimulated, without producing any effect. In the experiments yielding positive results, the output of ACh. per shock lay between 0·000002 and 0·00001μg., being thus well below that found in the perfusion experiments. Feldberg & Vartiainen only record one experiment made with natural circulation, and in this the concentration of ACh. in the venous blood from the ganglion rose during preganglionic stimulation to 1 in $2 \cdot 5 \times 10^7$, which was within the limits of concentration obtained, during similar stimulation, in a perfusion effluent. Since, however, blood flows from the venous cannula, with natural circulation, very much more slowly than the fluid with artificial perfusion, they clearly obtained a correspondingly less rapid output of ACh. Substantially, therefore, my positive results confirm theirs; but my general experience shows, in addition, that the appearance of ACh. in the venous outflow from the ganglion, as a result of preganglionic impulses, is not always to be detected when the ganglion retains its natural circulation.

DISCUSSION

The result of my experiments is to confirm those of Feldberg & Gaddum, and particularly those of Feldberg & Vartiainen. In contrast to the experience recorded by Lorente de Nó, I have not observed any continued or significant output of ACh. from the perfused ganglion without stimulation; I have never failed to observe its prompt appearance in response to, and in satisfactory coincidence with, periods of stimulation of the preganglionic nerve; and I have never detected any output during stimulation of either the vagus trunk or the sympathetic postganglionic branches, or in the periods succeeding such stimulation. In general, in my experiments with artificial perfusion of the ganglion, ACh. has regularly appeared in the venous effluent when the preganglionic nerve was stimulated, and only then. It failed so to appear only in one case, in which special measures had been taken to disturb the regular course of the perfusion. When the ganglion and the surrounding tissues have become oedematous, local variations in the resistance to perfusion might change the distribution of the flow, so that at one time the surrounding tissues, and at another those of the ganglion itself, might make the predominant contribution to the venous outflow; and one might expect such a changing incidence of the main perfusion stream to be more prominent with a slow perfusion at low pressure. It seemed possible that irregularities of this kind might be concerned in the capricious results recorded by Lorente de Nó, especially as he appears to have perfused under lower pressures than have generally been used by me, and by my predecessors in this line of investigation. My own attempts to produce such irregular conditions, however, have not on the whole been successful. In the one case mentioned, the output of ACh. failed when the pressure and perfusion rate were suddenly reduced; but in other cases I still observed a close correspondence between its appearance and the periods of preganglionic stimulation, even when pressures lower than those recorded by Lorente de Nó were employed. My difficulty, indeed, has been, not to obtain such coincidence, but to find conditions leading to a delayed or irregular output of ACh., or to its appearance with stimulation applied to nerves other than the preganglionic, as described by Lorente de Nó.

If **Lorente de Nó**'s attribution of the release of ACh. to injury of the ganglion were correct, it would be expected that perfusion with diluted blood instead of simple Locke's solution, and the consequent avoidance of any significant oedema, would be unfavourable to its regular appearance. My use of this method shows, on the contrary, that these conditions produce a remarkable uniformity, in different experiments and at different stages in the same experiment, of the amounts of ACh. liberated by the same number of preganglionic volleys. A ganglion so perfused shows, further, no significant difference from the corresponding normal ganglion, when examined histologically at the conclusion of an experiment. I did not, indeed, employ the same elaborate precautions as **Lorente de Nó** to avoid slight cooling or drying of the ganglion during the preliminary dissection. On the other hand, I regularly completed the dissection in 30 or 40 min., with care to avoid direct manipulation of the ganglion, in comparison with the 4–5 hr. required by **Lorente de Nó** for dissection under the microscope, in those experiments in which he attributed the smallness of the release of ACh. to the preservation of practically normal conditions in the ganglion.

My results thus give no support to the suggestion that the release of ACh. from the perfused ganglion is due merely to injury, apart from the arrival of preganglionic impulses at the synapses. On the other hand, they do not justify the assumption that the ACh. liberated at the synapses can escape, under the protection of eserine, into the blood circulating through the vessels of a ganglion which is otherwise perfectly normal. When the ganglion was allowed to retain its natural circulation, I detected the appearance of ACh. in the venous blood in response to preganglionic stimulation in only two out of five experiments, and then in relatively small amounts. One experiment of this kind was recorded by **Feldberg & Vartiainen** [1934] as evidence that the liberation could occur with natural blood supply, but a series would have been needed to show that it does so regularly; and, in fact, Dr **G. L. Brown** informs me that in subsequent experiments of this kind, which he made with Dr **Feldberg** in another connexion, positive results were not obtained in all cases, in conformity with my own findings. It is perhaps significant also that, in the largely analogous case of the voluntary muscle responding to motor nerve impulses, **Dale** *et al.* [1936] failed to detect any ACh. in the venous blood when the muscle retained its natural circulation under eserine, though it always appeared in the venous effluent with saline perfusion. It may, therefore, be regarded as not improbable, that some degree of departure from perfectly normal conditions may be required to enable ACh., liberated at the synapses in the ganglion, to escape into the circulation so as to be detected in the fluid leaving the vein. It may be that eserine, in doses tolerable by the whole animal, fails completely to inactivate the cholinesterase concentrated at the normal synapses [cf. v. Brücke, 1937]. On the other hand, as Feldberg & Vartiainen [1934] point out in discussing the action of eserine, the function of the cholinesterase in the ganglion may be to prevent the diffusion of excess of ACh. away from the point of its liberation at the synapse, rather than to remove it, by destruction at the site of its release, during the refractory period of the ganglion cell. There may be some other mechanism for such removal, effective under completely normal conditions. Normally most of the liberated ACh. may be immediately refixed in the inactive complex from which an impulse has released it; and the recent description by Mann *et al.* [1938] of the aerobic synthesis of an "acetylcholine-precursor" by brain tissue, suggests a method by which this may occur. Some degree of failure of normal oxygenation, or some increase of the permeability of the tissue above the normal, insufficient in either case to interfere with the transmission of excitation at the synapse, may, then, be necessary to enable the liberated ACh. to appear in the venous effluent in a quantity sufficient for detection and measurement. The question at issue, however, is not whether such slight abnormality is necessary to enable the liberation of ACh. to be detected, but whether, as Lorente de Nó appears to suggest, it is in itself the sufficient cause of such liberation. In my experiments it has not been so. The slight abnormality due to perfusion, if it exists, is one which can be regularly reproduced; it does not by itself lead to the appearance of ACh. in the venous fluid in significant amounts; it simply creates the conditions under which ACh. always appears in the effluent when preganglionic impulses reach the synapses, but never in their absence.

Lorente de Nó's experiments were made to control certain of those made by Feldberg & Gaddum [1934] and Feldberg & Vartiainen [1934], and his discussion deals essentially with the differences between his results and theirs, and his consequent doubt of the significance of their findings for the theory of transmission of excitation by ACh. at ganglionic synapses. The case for that theory cannot, however, be fairly assessed without consideration of additional points of evidence, of which Lorente de Nó's argument takes no account, and with which, indeed, it is at some points inconsistent. Brown & Feldberg [1936*a*] found that degeneration of the preganglionic fibres causes the disappearance of nearly all the ACh. which the normal ganglion yields to extraction, and that, after such degeneration, the perfused ganglion does not, like the normal ganglion, liberate ACh. in response to K ions, though these still effectively stimulate the ganglion cells. More recently [MacIntosh, 1938] I have shown that this disappearance of ACh. takes place in the 3 days following preganglionic section, in close accordance with the failure of transmission of excitation through the ganglion, at a time when, as Bacq & Coppée [1938] showed in parallel experiments, conduction in the preganglionic fibres is still maintained. At a time, therefore, when both the preganglionic fibres and the ganglion cells appear to be functionally intact, acetylcholine has almost disappeared from the ganglion and transmission across the synapses no longer occurs. Brown & Feldberg [1936*c*] further found that a normal ganglion will discharge up to five times its initial store of ACh. during continuous stimulation by preganglionic volleys for some hours, and still retain its original store undiminished. The selective action of certain poisons such as curare [Brown & Feldberg, 1936*b*] in rendering the ganglion cells completely insensitive to ACh. and to preganglionic impulses, while leaving them normally responsive to stimulation by K ions, must further be taken into account. It would be difficult to bring any of this evidence into intelligible relationship with Lorente de Nó's suggestion, that the ACh. liberated from a perfused ganglion is a product of the metabolism of, or of injury to, the ganglion cells, having no connexion with the transmission of excitation at the synapses.

SUMMARY

1. A method is described for the perfusion of the superior cervical ganglion with diluted blood.

2. Ganglia so perfused remain in excellent physiological condition and present no evidence of damage on histological examination.

3. Such ganglia do not liberate ACh. spontaneously, or on stimulation of the vagus or of the postganglionic sympathetic fibres: but ACh. is regularly liberated from them on stimulation of the preganglionic nerve. The liberation of ACh. begins with such stimulation, and ceases promptly after it.

4. The original findings of Feldberg & Gaddum and of Feldberg & Vartiainen are thus confirmed, and the significance in relation to them, of other evidence for the chemical transmission of excitation in the ganglion, is discussed.

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THE RATE OF ADAPTATION OF CUTANEOUS NERVE ENDINGS IN THE FROG

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Studies of the action potentials in sensory nerve fibres have led to a grouping of sensory endings as slowly adapting and rapidly adapting, the former giving a long-continued and the latter a brief discharge to a persistent stimulus. Many of the receptors in the frog's skin are of the rapidly adapting type, the discharge to a continuous stimulus lasting less than 0·1 sec.; others adapt somewhat more slowly (the discharge lasting from 1 to 100 sec.), while the stretch receptors in mammalian muscle, lung and other organs adapt still more slowly, the discharge usually continuing many minutes or even several hours. It is generally assumed that these differences express the constitutional properties of the nerve endings, but there is another possibility which has never been fully explored. This is that the more rapid failure of the discharge is due merely to rapid failure of the condition which acts as the immediate stimulus to the ending. A small weight resting on the skin produces a continued deformation of the skin, and in that sense the tactile stimulus may persist; but owing to yielding and readjustment of the tissues the deformation of the sensory nerve endings might occur only when the weight is first applied. In fact the rapid adaptation of the cutaneous endings might be due merely to a rapid regaining of their shape or position in spite of a continued pressure on the skin.

To test this point we have made preparations of the frog's skin in which we could identify a tactile ending giving a brief discharge to light pressure and have then stimulated it by stretching the skin laterally. Direct observation of the skin structures makes it appear unlikely that there will be any tendency for the endings to return to their normal position while the stimulus is in being. Since we find that rapid adaptation is still a feature of the nervous discharge, we conclude that it is due to the properties of the sensory endings and not of their surroundings.

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METHOD AND APPARATUS

It was essential to record the sensory discharge from single endings (or rather the endings of a single nerve fibre) without the risk of confusion from other fibres which might come into play when the stimulus was altered. To satisfy this condition we have used the antidromic preparation described by Adrian *et al.* [1931]. This preparation takes advantage of the fact that in the frog a few (usually only one, sometimes two) of the fibres in the dorsal cutaneous nerve branch near the cord and send their branches into both the dorsal and lateral cutaneous nerves and less frequently to one of the divisions of the ventral cutaneous nerve. A stimulus to the skin area supplied by one branch, e.g. the lateral, will send impulses towards the cord along this branch and the impulses will also spread down the other branches, travelling in them *towards* the skin as in Fig. 1 B. Thus by recording the antidromic discharges in, for example, the dorsal branch, we can record the activity of only one or two of the sensory fibres supplying the stimulated area.

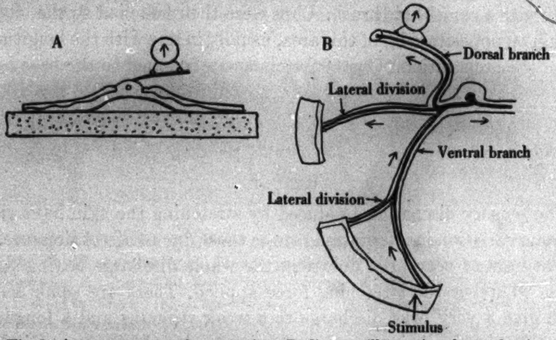

Fig. 1. A, arrangement of preparation. B, diagram illustrating the production of antidromic impulses in the dorsal cutaneous nerves.

The arrangement of the preparation is shown in Fig. 1 B. The frog was pithed, opened along the median ventral line and eviscerated, and the spinal cord and sympathetic system completely destroyed. The body wall (with abdominal musculature as well as skin) was then spread out on a cork plate, and one of the dorsal cutaneous nerves which had been tied off and divided close to the skin was placed on the recording electrodes and examined for the presence of antidromic discharges when the skin was stimulated by touch. It was not difficult as a rule to find a dorsal nerve with only one fibre giving the antidromic discharge. In each preparation usually three to four, sometimes as many as five, nerves gave single antidromic responses to stimulation of the skin. The skin areas which gave the response were found most frequently close to the dorsal septa, more infrequently on the lateral, and least frequently on the ventral surface. In general the area giving the antidromic response appears to lie within the segmental area on the skin corresponding to the particular spinal nerve under examination, but occasionally it lies in an adjacent segment.

The usual procedure was to select a dorsal cutaneous nerve giving antidromic responses in a single fibre when a particular area was touched lightly with a camel-hair brush. Clips were then fastened to the edges of the skin on opposite sides of this area, usually in line with the longitudinal axis of the preparation. One of the clips was fastened to the base board, and a weight of 25, 50 or 100 g. was attached to the other, so that it could be dropped freely through about $\frac{1}{2}$ cm., pulling the two clips apart and exerting a sudden stretch on the skin between.

RESULTS

The sensory discharges produced by stretching the skin have shown the same variation in adaptation rate as those due to direct pressure. The majority are of very brief duration, the whole discharge lasting $\frac{1}{10}$ sec. or less whatever the stretching force applied. There are some endings which give a very brief discharge to a weak stimulus and a longer one when the stimulus is increased, and some again which give a discharge continued over a period of from 1 sec. to 2 min. even when the weakest of the three stretching forces is applied, although the responses to interrupted stimulation by touch are of brief duration and the response on release after complete adaptation is brief. The relative frequency with which endings of various adaptation rates were found can be seen in Table I.

TABLE I. Duration of discharge in response to stretch (distribution of times for thirty-four endings)

	No. of observations			
Duration	25 g.	50 g.	100 g.	Total
No response*	2	1	1	4
Less than 0·009 sec.	10	7	5	22
0·01–0·09 sec.	12	14	15	41
0·1–0·9 sec.	2	2	3	7
1–9 sec.	5	1	0	6
10–100 sec.	2	7	7	16
100–1000 sec.	0	0	1	1
Total	33	32	32	97

* Response to touch and sudden stretch by hand elicitable.

The table suggests that the responses are not distributed in a continuous series, but are divided into two distinct groups with rapid and with slow adaptation. The division does not appear in the responses to the 25 g. stretch, but with the heavier weight the discharge tends either to be shorter than 0·1 sec. or else longer than 10 sec. The endings adapting slowly (giving discharges lasting from 10 to 100 sec.) were 17·5% of the total number.

Among the rapidly adapting responses, the total number of impulses set up by a single stimulus varied from 1 to 12. The highest frequency (during the first 0·1 sec.) was approximately 180 per sec. The duration of discharge showed no clear dependence on the magnitude of the force applied. Weights as heavy as 400 g. were used without an appreciable effect upon the duration. A sudden application of the stimulus was always necessary to produce the response.

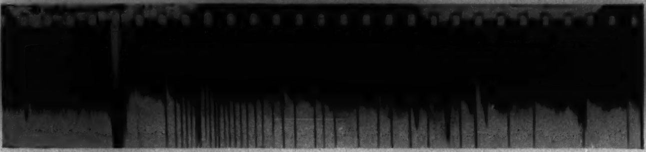

Fig. 2. Record showing the response of a tactile ending to stretching the skin. The ending is of the slowly adapting type. Stretch produced by 100 g. weight applied suddenly (moment of application marked by large excursion of base-line). Frequency is 180 per sec. during the first 0·1 sec. of the discharge. Time marker (above) gives 0·6 sec. interval from one white area to the next.

For the slowly adapting responses the duration of the discharge increased as the stretching force was increased and the response was much less dependent upon the rate of increase of the stimulus. Adaptation times varied, for the 25 g. weight, from 1·45 to 23 sec.; for the 50 g. weight, from 8 to 33 sec.; for the 100 g. weight, from 18 to 110 sec. The record shown in Fig. 2 illustrates the slowly adapting type of response.

(*a*) *Direction of stretch.* In several preparations the sensory discharge was evoked by stretching the skin in several different directions. A stretch in the long axis of the body might cause a different amount of deformation of the nerve endings from one across the body, and if this were so the intensity and duration of the discharge might differ. It was found, however, that there was no significant difference except in one preparation where the conditions were not kept otherwise the same. It appears, therefore, that the sensory endings from a single fibre do not lie mainly in one direction and are likely to be deformed by stretch whatever the direction in which it is applied.

(*b*) *Are the more slowly adapting endings in the skin or muscle?* Endings adapting slowly to pressure have been found before in preparations of the frog's skin without muscular tissue, but in the present experiments it seemed possible that the endings having the longer adaptation times might be stretch receptors in the abdominal muscles and not tactile endings in the skin covering these muscles. To eliminate this possibility a few experiments were made in which the ventral branch of the nerve was used for recording the antidromic discharges and the stretch was applied to the dorsal skin after complete separation from the muscles. Slowly adapting responses were still obtained with this arrangement; it is clear, therefore, that they can be produced by stretching endings which are in the skin. With a few of the slowly adapting endings, records were made of the response to considerable pressure as well as that to stretch. There was no significant difference in the durations of the discharges.

(*c*) *Can the endings recover their normal position whilst the stimulus is still applied?* Segments of skin from the abdomen were stained vitally with methylene blue and examined under the microscope when the skin was stretched in various directions to see whether or not this mode of stimulation does eliminate all possibility of the tactile ending's resuming its position while the stimulus is still in being. Slight changes in tension were found to alter materially the positions of the nerve fibres, their endings, the surrounding tissue and blood corpuscles, and no one of them exhibited a tendency to return to the resting position until the tension was relaxed. Evidently yielding and readjustment of the tissues do not occur to any great extent, and it is extremely unlikely that the rapid adaptation times are due to it. The observations in the following sections on the effect of ions on the adaptation rate lead to the same conclusion, namely, that differences in adaptation are due to the constitutional properties of the nerve endings.

(*d*) *Effect of ions.* If the rate of adaptation is not determined merely by the yielding of the tissues, it should be possible to modify it by changing the ionic content of the fluid round the endings. Talaat [1933] has already reported that slow adaptation times could be produced by removal of Ca ions in the frog's skin, and we have confirmed this in a number of preparations using Talaat's proportions of eight parts of Ca-free Ringer to two parts of 1·2% Na oxalate. The results of one such experiment are given below (Table II).

TABLE II. Duration of discharge from a rapidly adapting tactile ending before and after reduction of Ca in Ringer

	Duration of discharge (sec.) (50 g.)	
Solution	Stretch	Pressure
Ringer	0·01–0·1	0·01–0·1
Ca-free Ringer and Na oxalate (15 min.)	65	120

The reverse change, producing a more rapid adaptation by increasing the Ca content of the fluid, is illustrated in the following experiment. This change as well as the previous one is reversible.

TABLE III. Duration of discharge from more slowly adapting tactile endings before and after increase of Ca in Ringer

	Duration of discharge (sec.)		
Solution	25 g.	50 g.	100 g.
Normal Ringer (Ca 0·012 %)	1·3	12	21
Ringer with increased Ca (Ca 0·092 %), 1 hr.	0·15	0·72	14
Return to normal Ringer	12	26	37
Return to Ringer with increased Ca, 2 hr.	No response*		

* Responses to touch could still be elicited.

	Duration of discharge (sec.) (50 g.)	
Solution	Stretch	Pressure
Normal Ringer (Ca %)	90	50
Ringer with increased Ca:		
30 min.	100	110
55 min.	1 impulse	0·01–0·1

These pronounced changes produced by excess or deficiency of calcium show that the duration of the discharge from the skin endings would be governed to some extent by the concentration of Ca near them. Evidently the ionic concentration in the tissue fluids might be an important factor in deciding the rate of adaptation of a given ending. There is, however, no clear evidence either for or against the existence of localized differences in concentration in the neighbourhood of different endings.

SUMMARY

1. The differences in the rate of adaptation to the stimulus which are found with different types of nerve ending might be due in part to purely mechanical factors. In particular the rapid adaptation of the tactile endings in the skin might be due to a rapid yielding of the tissues which prevents the stimulus from causing a persistent deformation of the endings. It is found, however, that:

2. The discharges produced in sensory nerve fibres of the frog by stretching the skin differ very little from those produced in the same fibres by touch or pressure: endings which give a brief discharge to moderate and strong pressure give an equally brief discharge when stimulated by stretching the skin.

3. Since there can be little opportunity for the endings to revert to the original position whilst the stretch is maintained, the brevity of the discharge cannot be due merely to mechanical changes in the surrounding tissues, and it follows that the rate of adaptation to the stimulus expresses some genuine constitutional property of the nerve ending.

4. We have confirmed Talaat's observation that the duration of discharge of the cutaneous endings may be increased by a reduction of Ca, and have found that normally long durations can be reduced by an excess of Ca.

5. The two types of response in the tactile endings have been found to be distributed not in a continuous series but to be divided into two distinct groups or discrete types of response, the rapidly adapting type with a total duration of 0·1 sec. or less and the slowly adapting with a duration longer than 10 sec. Intermediate durations are found, but the majority of the endings fall into one group or the other.

The authors wish to express their gratitude to Prof. E. D. Adrian for guidance and advice. They are also indebted to Dr B. H. C. Matthews for much valuable help.

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FUNCTIONAL IMPAIRMENT OF THE ANTERIOR PITUITARY GLAND PRODUCED BY THE SYNTHETIC OESTROGENIC SUBSTANCE 4:4′ DIHYDROXY-α:β-DIETHYLSTILBENE

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A HIGHLY active, synthetic oestrogenic substance 4:4′-dihydroxy-α:β-diethylstilbene (diethylstilboestrol) has been described by Dodds, Golberg, Lawson and Robinson [1938], the effects of which are similar to those produced by the naturally occurring hormone oestrone [Dodds, Lawson & Noble, 1938]. The synthetic substance was found to be more than twice as active as oestrone when judged by the dosage required to produce oestrus in ovariectomized rats. That synthetic oestrogenic substances may exert an action similar to oestrone has also been shown by Wolfe [1936], who found that the histological changes which appear in cells of the anterior pituitary after ovariectomy may be restored to normal as readily by a synthetic substance as by oestrone. The subcutaneous implantation of crystals of diethylstilboestrol may also cause alterations in the rate of body growth and endocrine function [Noble, 1938]. In the following experiments large doses of diethylstilboestrol have been injected in oil solutions into rats and the effects on anterior pituitary function determined.

METHODS

The active substance was dissolved in sesame oil and injected subcutaneously into adult white rats of the Wistar strain. In one group of experiments a total dose of 10–30 mg. diethylstilboestrol was administered in divided doses or by daily injections to male and female rats. Another series of female rats received a total dose of 200 mg. in daily injections of 10 mg. for 20 days. The animals were killed at various intervals and the endocrine organs dissected out and weighed. Hypophysectomy was performed under ether anaesthesia by the retro-pharyngeal approach.

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RESULTS

Effect of diethylstilboestrol

Male rats. In Table I the effects produced in nine male rats are recorded. In Table II the changes produced in organ weights following hypophysectomy are shown for comparison. The effects of the oestrogenic substance were reflected mainly in a lowering in weight of the seminal vesicles and prostate. The testes were reduced in size in some cases, but atrophy of the secondary sex organs may be present with normal-sized testes. Enlargement of the pituitary gland has been noted, but the secondary changes in the gonads do not appear to be directly related to alterations in pituitary weight. The adrenal glands were increased in weight. These changes, when compared with those in Table II due to hypophysectomy, indicate that the gonadotropic activity of the pituitary may be completely suppressed, with a resulting atrophy of the sex organs. Atrophy as marked as in hypophysectomized animals was more readily

TABLE I. Effect of diethylstilboestrol on endocrine weights in male rats

					Weight per 100 g. body weight				
Rat no.	Total dose mg.	Injected on days	Killed on days	Weight change g.	Adrenals mg.	Seminal vesicles mg.	Prostate mg.	Testes mg.	Pituitary mg.
17	10	1, 5	15	+19	35	25	72	783	4·7
16	10	1, 5	22	+24	40	17	87	1214	4·5
18	15	1, 5, 12	19	+ 1	37	40	117	1226	6·0
19	20	1, 5, 12, 19	26	0	46	26	73	391	5·0
20	25	1, 5, 12, 19, 26	33	+17	31	33	75	621	10·1
21	30	1, 5, 12, 19, 26, 33	40	+13	47	32	67	641	8·1
56	10	1 to 14	15	− 4	35	39	67	1281	6·4
58	15	1 to 21	22	−27	48	21	50	388	8·6
57	20	1 to 28	29	−12	36	27	44	319	6·9
Average for control rats					13	284	294	1215	3·7

TABLE II. Effect of hypophysectomy on endocrine organs of male rats

		Weight per 100 g. body weight			
Time after hypophysectomy weeks	No. of rats	Adrenals mg.	Seminal vesicles mg.	Prostate mg.	Testes mg.
1	4	12·0	52	120	1204
2	4	12·0	56	109	854
4	5	8·0	42	87	366
6	2	5·0	34	54	273

shown in the seminal vesicles and prostate than in the testes. The adrenal-stimulating factor of the pituitary would not seem to be inhibited in that the adrenal weights were increased. Some alteration in body weight was apparent and could be explained by an inhibition of the growth hormone of the anterior lobe [Zondek, 1936; Noble, 1938].

Female rats. Table III contains the results from a series of nine female rats treated in the same way as the male animals, and Table IV the weights obtained in hypophysectomized female rats. The changes in weight of the ovaries are similar to those occurring after pituitary removal. The adrenal glands were enlarged in the female rats as in the males. The pituitary was enlarged in most animals, but atrophy of the gonads, as in the male, was not directly related to pituitary size. The uteri were enlarged and distended with fluid due to a direct action of the synthetic substance, and vaginal smears of these animals showed a persistent oestrus or pro-oestrus condition.

TABLE III. Effect of diethylstilboestrol on endocrine weights in female rats

Rat no.	Total dose mg.	Injected on days	Killed on days	Weight change g.	Weight per 100 g. body weight Adrenals mg.	Ovaries mg.	Uterus mg.	Pituitary mg.
22	10	1, 5	15	+10	62	28	324	7·7
23	10	1, 5	22	+ 6	35	26	212	6·3
24	15	1, 5, 12	19	+ 3	59	24	271	8·5
25	20	1, 5, 12, 19	26	+ 8	43	15	186	8·4
26	25	1, 5, 12, 19, 26	33	+ 6	69	20	274	7·2
27	30	1, 5, 12, 19, 26, 33	40	+18	58	10	250	10·2
59	10	1 to 14	15	−11	48	41	409	7·0
60	15	1 to 21	22	−17	47	20	331	10·9
61	20	1 to 28	29	−12	43	19	392	14·4
		Average for control rats			26·5	32	287	6·0

TABLE IV. Effect of hypophysectomy on endocrine organs of female rats

Time after hypophysectomy weeks	No. of rats	Weight per 100 g. body weight Adrenals mg.	Ovaries mg.	Uterus mg.
1	4	28·0	24	142
2	6	23·0	22	129
4	4	14·0	17	117
6	3	8·0	15	82

The effects of much larger doses—200 mg. given in 10 mg. doses for 20 days—may be seen in Table V. The action of such large doses appeared to last for a long time. Seven weeks after the last injection the ovaries were still atrophic and covered with fibrous adhesions. The adrenals and pituitary had returned to a more normal weight. The gradual

TABLE V. Effect of 200 mg. diethylstilboestrol administered over 20 days on the endocrine organs

Rat no.	Killed, days from last injection	Weight change g.	Weight per 100 g. body weight			
			Adrenals mg.	Ovaries mg.	Uterus mg.	Pituitary mg.
220	3	−10	54	62	1394	15·1
221	4	−28	57	36	611	11·5
227	14	−20	34	24	533	10·6
230	14	−44	41	15	841	21·5
224	28	−25	39	7	361	9·2
226	28	+ 3	20	7	400	9·8
223	49	+ 5	27	14	408	7·9
231	49	− 5	22	11	616	10·2

decrease in weight of the ovaries is probably related to the initial stimulation of the corpora lutea, since Robson [1937] has found that oestrone may maintain the corpora lutea even after hypophysectomy. In the animals killed 4 days after the last injection it was found that the mammary glands and nipples were fully developed and milk could be expressed without difficulty.

Effect of gonadotropic hormones

Since the changes following administration of diethylstilboestrol appeared comparable to the withdrawal of gonadotropic activity due to pituitary removal it was of interest to determine how treated rats would respond to gonadotropic preparations. Eight normal rats received a total each of 25 mg. diethylstilboestrol to produce diminished pituitary function. Two of these were completely hypophysectomized and killed after 6 days. Two were used as controls. Two received a total of 100 R.U.

TABLE VI. The effect of gonadotropic hormones after diethylstilboestrol injections

Rat no.	Total dose stilboestrol over days 1–31 (mg.)		Killed on day	Weight change g.	Weight per 100 g. body weight			
					Adrenals mg.	Ovaries mg.	Uterus mg.	Pituitary mg.
30	25	—	37	−27	58	18	324	13·4
31	25	—	37	− 5	58	13	332	10·1
32	25	Hypophysectomized on 32nd day	38	−19	36	Fibrosis and pyometria		—
33	25	Hypophysectomized on 32nd day	38	−35	33	17	296	—
28	25	100 M.U. Antex over 32nd–36th day	37	+ 3	41	76	418	12·1
29	25	100 M.U. Antex over 32nd–36th day	37	− 1	47	82	434	12·8
34	25	100 R.U. Pregnyl over 32nd–36th day	37	− 3	50	84	295	14·9
35	25	100 R.U. Pregnyl over 32nd–36th day	37	−12	59	54	Pyometria	15·1

pregnancy urine extract "Pregnyl" each over 5 days and were killed on the 6th day, and the two others received a total of 100 M.U. pregnant mare serum extract "Antex" over a similar period. The organ weights are recorded in Table VI. The animals hypophysectomized showed a decrease in adrenal weight which would suggest that the adrenotropic hormone of the anterior pituitary is not inhibited to the same extent as the gonadotropic. The effect of pregnant mare serum on the ovary was similar to that obtained in hypophysectomized rats under comparable conditions. The effect of pregnancy urine differs completely from that in hypophysectomized rats in that a marked increase in weight of the ovaries was produced.

Water metabolism

The necessary role of the anterior lobe of the pituitary in the maintenance of diabetes insipidus is well established [Richter, 1934; Fisher & Ingram, 1936; Dodds, Noble & Williams, 1937]. Since removal of the anterior lobe cures this condition, various observations have been reported where large doses of oestrone have been administered clinically to inhibit anterior pituitary function. Recent reports indicate that the diuretic substance secreted by the anterior lobe is responsible for the maintenance of polyuria, although some part is played by the thyroid through the thyrotropic principle [Fisher & Ingram, 1937; White & Heinbecker, 1937]. Some clinical cases would support the idea that oestrone will inhibit the action of the anterior lobe substances involved in water metabolism [Shapiro, 1938; Fisher *et al.* 1938]. The water metabolism of twenty-six rats has been estimated during the course of injections of large doses of this synthetic oestrogenic compound. The animals were fed on dry compressed food and the 24 hr. water intake and urine output measured. Although evidence of deficient anterior lobe gonadotropic function has definitely been produced, the changes in urinary secretion have been slight. From Table VII it can be seen that a consistent decrease in water intake and a gradual increase in urinary output were produced. Food intake was not measured but appeared to be less than in the control period. The changes observed do not support the view that the diuretic substance of the anterior lobe is suppressed, since under these conditions one would expect a diminished urinary output due to overaction of the antidiuretic posterior lobe principle. The effect on animals with diabetes insipidus has not yet been investigated. The two rats hypophysectomized exhibited a typical postoperative transitory polyuria similar to that found in normal rats [Richter, 1934; Dodds, Noble & Williams, 1937].

TABLE VII. Fluid balance following diethylstilboestrol injections

Rats no. and sex	Average weight g.		24 hr. average per rat c.c.					
			Control period	Days 1–7	Days 8–14	Days 15–21	Days 22–28	Days 29–35
16.17 ♂	104	Water intake	9·5	11·0	13·5	—	—	—
		Urine output	0·8	1·2	1·6	—	—	—
18.19 ♂	149	„	15·6	11·8	14·0	—	—	—
			0·7	1·3	1·4	—	—	—
22.23 ♀	127	„	16·0	13·7	15·2	—	—	—
			1·5	3·7	5·0	—	—	—
24.25 ♀	123	„	15·6	11·9	11·7	—	—	—
			1·1	1·6	1·9	—	—	—
28.29 ♀	167	„	21·5	16·9	15·5	—	—	—
			2·4	3·8	3·3	—	—	—
30.31 ♀	141	„	25·1	16·0	17·5	—	—	—
			2·0	3·0	4·6	—	—	—
32.33 ♀	150	„	23·3	20·7	22·0	—	—	—
			2·2	5·0	5·0	—	—	—
34.35 ♀	136	„	20·5	16·7	19·4	—	—	—
			1·9	3·5	5·2	—	—	—
20.21 ♂	138	„	16·7	11·7	14·2	16·7	18·7	21·4
			1·3	1·8	1·7	2·7	3·2	4·9
26.27 ♀	113	„	18·3	11·7	13·0	13·7	15·2	17·6
			1·5	1·7	2·4	2·8	2·7	4·3
57.58 ♂	154	„	20·0	12·8	13·9	12·5	16·0	—
			1·5	1·3	2·2	2·9	4·9	—
60.61 ♀	136	„	16·1	13·6	14·0	15·9	17·2	—
			2·4	3·0	3·3	4·8	8·2	—

SUMMARY

Large doses of diethylstilboestrol injected into rats in oil solutions were followed by atrophy of the testes, prostate and seminal vesicles in the male, ovarian atrophy in the female and an increase in weight of the adrenals and pituitary of both sexes.

Female rats after synthetic oestrogenic treatment responded by an increase in ovarian weight to pregnant mare serum and pregnancy urine extracts.

The changes produced in water balance did not indicate that any alteration in the secretion of the diuretic principle of the anterior pituitary had occurred.

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NISSL GRANULES IN "FATIGUED" NERVE CELLS

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Nissl granules are held to vary in size, number and arrangement in accordance with the functional state of the neurone, and to undergo chromatolysis in a fatigued or injured nerve cell [Mann, 1895]. Wright [1900] demonstrated chromatolysis, which he attributed to the action of anaesthetics upon the living nerve cell. The present experiments were undertaken to determine whether prolonged stimulation of nerve cells affected the staining properties of the Nissl granules.

Method

The blood pressure was recorded in decerebrate cats during continuous stimulation of the right splanchnic nerve for a period varying from 2 to 5½ hr., through Collison's [1934] fluid electrodes, from an induction coil, at a rate of 34 per sec. The right suprarenal gland was previously removed, to avoid secondary effects due to outpouring of adrenaline. The length of the period of stimulation was determined by testing, at intervals, for a rise in blood pressure in response to an increase in the strength of stimulus. As soon as the test failed to give any further rise, the right coeliac ganglion was quickly removed, fixed in 7 p.c. formalin and embedded in paraffin. Sections were stained with toluidine blue or haematoxylin and Biebrich scarlet. Sections, for use as controls, were made of the left coeliac ganglion and of ganglia from cats subjected to no experimental procedure. Ganglia, the fixation of which had been deliberately delayed, ½ hr. or longer, were also used for comparison.

Results

The nerve cells in the control sections stained with toluidine blue showed well-defined Nissl granules. If, however, fixation was delayed, the cells showed absence or blurring of the granules. They were distorted, with smaller nuclei sometimes eccentric, and nucleoli less well stained than in the controls fixed without delay.

In the cells of stimulated ganglia from five successful experiments no such changes were found. Sections of these ganglia, stained with haematoxylin and Biebrich scarlet showed that the tissue was slightly more vascular than in their controls.

DISCUSSION

The Nissl granules may represent a reserve of food material. Da Fano [1923] found changes in the Nissl substance associated with deficiency disease. No histological changes indicating a depletion of this reserve, during prolonged stimulation of the nerve cell, are shown in the present experiments, although cell injury, caused by delayed fixation, was accompanied by gross changes. The failure of the vaso-constrictor response after prolonged stimulation, which was used as a test for fatigue in the postganglionic vaso-constrictors, is possibly the cause of the increased vascularity of the stimulated ganglia. This test does not necessarily imply fatigue in the ganglion cells themselves, which were stimulated through the preganglionic fibre. Brown & Feldberg [1936] have shown that synthesis of acetylcholine, within the ganglion, can occur under similar conditions. It seems possible, therefore, that "fatigue", i.e. exhaustion of function, associated with gross histological changes, does not occur in autonomic nerve cells, apart from actual cell injury.

SUMMARY

Prolonged stimulation of autonomic ganglia produces no change in the appearance of the Nissl granules of the nerve cell.

My thanks are due to Dr E. E. Hewer for her advice and help.

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LIBERATION OF HISTAMINE AND FORMATION OF LYSOCITHIN-LIKE SUBSTANCES BY COBRA VENOM

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We have recently shown [1937*a*] that cobra venom causes the release of histamine from perfused lungs of cats and guinea-pigs. The experiments reported in the present paper concern the liberation of histamine from liver and lungs of other animals and the manner in which this effect may be produced.

One of the actions of cobra venom is to cause haemolysis; this is not a direct effect of the venom on the red blood corpuscle but results from its action on lecithin. Kyes [1904] in Ehrlich's laboratory found that if cobra venom is brought into contact with lecithin a haemolytic substance is produced. Delezenne & Fourneau [1914] showed that this substance is a derivative of lecithin. The cobra venom contains a lecithinase which splits off oleic acid from lecithin. The resulting rest, lysocithin, is powerfully haemolytic. It appeared conceivable that the formation of lysocithin might account for effects of the venom, other than haemolysis and that its formation in the tissues might make the cells permeable to their histamine. Although this theory has not hitherto been put forward in this form, observations of Belfanti [1925, 1928], Guerrini [1925] and Houssay and his co-workers [Houssay, 1930] suggest that the formation of lysocithin may play a dominant role in the symptomatology of snake venom poisoning. They showed that the injection of lysocithin into animals caused symptoms resembling in some respects those caused by snake venoms. On the other hand, Belfanti's results on the action of snake venoms on emulsions made from different organs seemed to exclude the formation of lysocithin in all the tissues tested (heart, liver, kidney,

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spleen) except brain. If the conclusions drawn from these experiments were correct the effects of snake venom on isolated perfused organs would be independent of the formation of lysocithin.

In our previous experiments on perfused lungs we have found that the venous perfusate after the injection of venom caused not only a rapid histamine-like contraction but sometimes also a delayed and slow contraction of the isolated jejunum of the guinea-pig. We have encountered a similar phenomenon in experiments on the effect of bee venom on the perfused guinea-pig's lung and dog's liver [1937*b*]. It was tempting to assume that lysocithin from lecithin or substances formed in the tissues from lipins other than lecithin might account for this slow contraction. A comparison of the unknown active principle with lysocithin was therefore indicated.

Our experiments are divided into two parts: the action of cobra venom on perfused lungs and livers and a comparison of some of the pharmacological effects of the active substance or substances formed by venom in perfused organs with those formed *in vitro* by venom on lecithin.

METHODS

We perfused the lungs and livers of dogs and monkeys (*Macacus rhesus*) with Tyrode solution using the method already described [1937*a*, *b*], the rate of perfusion being 6–12 c.c. per min. Whereas only a small portion of the dog's liver was used almost the whole of the monkey's liver, weighing 60–80 g., was perfused. The venous perfusate was assayed for histamine on a piece of guinea-pig's jejunum suspended in a 3 c.c. bath. Protein in the perfusate was roughly estimated by Esbach's method.

Extracts of organs. Saline extracts were made by grinding the organs with powdered quartz in Tyrode solution [for details see 1937*a*]. The extracts were boiled before testing with the exception of some of the monkey's liver which were also tested unboiled. Extracts from the monkey's liver were also made in absolute methyl alcohol. The organ ground with powdered quartz was repeatedly extracted with alcohol, about 10 c.c. being used per gram of tissue. The extract was filtered, taken to dryness on the water bath and re-extracted with absolute methyl alcohol. This was again filtered. The final filtrate was made up to such a volume that 1 c.c. corresponded to 1 g. of liver. For use, measured amounts of the extract were evaporated and the residue taken up in a given volume of physiological saline solution. Methyl alcoholic extracts made from monkey's liver after perfusion and the injection of large doses of cobra venom will be termed "envenomed liver".

Preparation of "lysocithin". Several batches of lysocithin were prepared by the action of cobra venom on egg lecithin which was made for us by our colleague, H. F. Holden. One gram of lecithin was emulsified and suspended in 1300 c.c. of buffered phosphate solution at *p*H 7·7. The solution contained 400 c.c. $N/5$ KH_2PO_4 and 70 c.c. 1·015 N NaOH per l. The suspension of lecithin was brought to 37° C. in a water bath and 0·8–1·2 g. of cobra venom dissolved in 20 c.c. of physiological saline solution was added. Samples of the mixture were at once tested in various dilutions for their capacity to haemolyse washed sheep red corpuscles, which are not haemolysed by cobra venom in any concentration but undergo immediate haemolysis with lysocithin. Further tests were made from time to time, and when no further increase in the titre of lysocithin was to be seen the mixture was taken to dryness on a boiling water bath and extracted by repeated rubbing up with pure absolute methyl alcohol. The methyl alcohol extract was filtered, taken to dryness and again taken up in absolute methyl alcohol. This extract was filtered and made up to 50 or 100 c.c. so that 1 c.c. of our extract contained lysocithin equivalent to 10 or 20 mg. of lecithin. Although this process of concentration involved evaporation on a boiling water bath for several hours, twice extracting with methyl alcohol and twice filtering, there was no appreciable loss of haemolytic activity. For use, the alcoholic solution was evaporated to dryness on a water bath and emulsified in physiological saline. The appearance of these emulsions varied somewhat. Two batches yielded a clear gelatinous watery solution, but more usually a fine opaque suspension which did not sediment and readily passed through a No. 1 Whatman filter paper without loss of activity was obtained.

We made no attempt to prepare pure lysocithin. Our extracts may, therefore, have contained other active substances formed by the action of cobra venom on lecithin or, since our sample of lecithin was probably not pure, from impurities present in it. For this reason, where we refer to our extracts of lysocithin we use inverted commas.

We shall express the amount of "lysocithin" as equivalents of pure lecithin. There was some variation in the activity of different batches. Comparison of the activity of "lysocithin" in different experiments and pharmacological tests were, therefore, always made with a single batch.

Pharmacological tests. Uterus of the virgin guinea-pig; jejunum of the young rat. The muscles were suspended in a 3 c.c. bath in oxygenated anaphylactic Ringer's solution (NaCl 0·9%, KCl 0·042%, $CaCl_2$ 0·012%, glucose 0·1%, $NaHCO_3$ 0·05%). *Cat's heart:* Gunn's modification [1913] of Langendorff's perfusion method was used. The water in the heating jacket was supplied from a large thermostat which was also used to warm the Ringer's solution (NaCl 0·9%, KCl 0·042%, $CaCl_2$ 0·024%, glucose 0·1%, $NaHCO_3$ 0·01%). The thermostat was supplied with a continuous flow of tap water from a constant level and the outflow to the water jacket was also supplied from a constant level in the thermostat. With this arrangement the temperature of the perfusing fluid, despite large variations in coronary flow, did not alter more than 0·5° C. during the course of an experiment. The reservoir of the cold Ringer's solution was 80–90 cm. above the heart and was oxygenated by passing "carbogen" (5% CO_2 in O_2) through a coarse porous filter immersed in it. The Ringer's solution was brought to a pH of about 7·5 (estimated by colorimetric comparison using phenol red as an indicator) by bubbling CO_2 through it. All injections were made in a volume of 1 c.c. directly into the aortic cannula. *Sheep's red corpuscles:* The cells were washed three times in 0·9% NaCl solution and used in a 5% suspension. The haemolytic system was 0·2 c.c. (0·1 c.c. of cells and 0·1 c.c. of the solutions tested which were put up in doubling dilutions). The tests were made at 37° C. in a water bath and the haemolysis times determined by continuous or frequent observations.

RESULTS

PART 1. EFFECTS OF COBRA VENOM ON PERFUSED LUNGS AND LIVER

In addition to the changes caused by the venom in these organs there appeared in the venous perfusate, protein, histamine, a substance (or substances) which causes a delayed and slow contraction of the guinea-pig's jejunum and, in the case of the liver, pigments.

A. *Identification of histamine*

The identification of histamine in perfusates and organ extracts was the same in all experiments. Since the liver contains acetylcholine and choline it was possible that part of the histamine-like activity on the gut of the liver extracts and perfusates might be caused by these substances. Acetylcholine was excluded by treating the solutions with alkali, which did not diminish their activity. This was to be expected since the conditions under which extracts and perfusates were obtained favoured hydrolysis of acetylcholine. The presence of choline in effective concentrations was improbable because the amounts of extract necessary for the assay usually corresponded to between 0·5 and 1 mg. of fresh tissue, and because perfusates could be tested in high dilution. Choline was excluded by the fact that the activity persisted after atropine.

The identification as histamine of the substance *in extracts* which caused rapid contraction of the gut rested upon pharmacological assays in which its activity was compared quantitatively with histamine in three different tests and upon the fact that histamine has been isolated from livers and lungs, identified chemically and made responsible for the histamine-like activity of extracts [Hanke & Koessler, 1924; Lucas, 1926; Best, Dale, Dudley & Thorpe, 1927]. The identification of this active substance *in perfusates* rested upon pharmacological assay by the same tests, and upon the demonstration that it was part of the tissue histamine, which diminished to the same extent as the activity in the perfusate increased. We shall therefore refer to it as histamine. In Fig. 1 is illustrated the assay of a sample of perfusate collected from a piece of perfused dog's liver in the second hour after the intraportal injection of 18 mg. of cobra venom. The stimulating action of the boiled perfusate on the guinea-pig's jejunum corresponded to that of histamine 1 : 500,000. In the upper tracings of Fig. 1 the response of the plain muscle to 0·2 c.c. of a 1 : 10 dilution of this sample is "bracketed" by responses to 0·2 c.c. of two histamine solutions, 1 : 6 million and 1 : 4 million. In the middle and lower tracings the effect of 0·5 c.c. of the undiluted sample is "bracketed" by responses to 0·5 c.c. of two histamine solutions 1 : 600,000 and 1 : 400,000. The middle tracings show the depressor action of these solutions on the cats' blood pressure by intravenous injection. The lower tracings show the pressor responses resulting from output of adrenaline in the same cat, after evisceration and ligature of the aorta and vena cava inferior distal to the suprarenals, the solutions

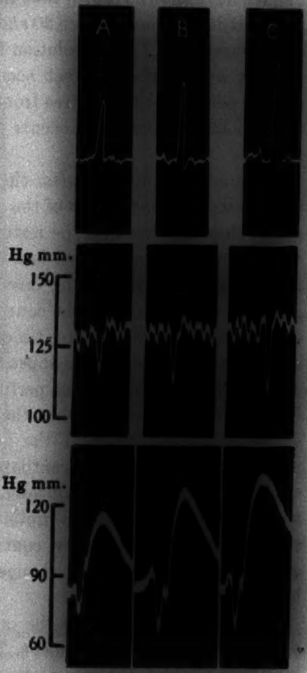

Fig. 1. Records from guinea-pig's jejunum (upper tracing) and cat's arterial blood pressure (middle and lower tracings). A and C, histamine; B, perfusate from envenomed dog's liver. (Details in text.)

being injected into a cannula tied into the central stump of the coeliac axis. It will be seen that in all these tests the undiluted perfusate was stronger than histamine, 1:600,000, and weaker than histamine, 1:400,000.

B. *Dog's lungs*

We have shown [1937*b*] that the histamine equivalent of the lungs in different dogs varies between 20 and 220 μg. per g.; that isolated lobes may be perfused with Tyrode solution for many hours without gross changes in their appearance or great accumulation of fluid in them; that the venous perfusate remains free from detectable amounts of histamine and that no loss of histamine occurs from the lungs during prolonged perfusion.

Two or three minutes after the injection of venom (10 mg. or more), the respiratory movements of the artificially ventilated lobes diminish or cease; they can, however, be restored by increasing the ventilation. The venous outflow slows down and may stop without diminution of the inflow; tremendous swelling occurs and the lungs become heavy and glassy in appearance. In the course of some 10 min., fluid, in an amount corresponding to two or three times the weight of the perfused lobes, may accumulate and some may appear in the trachea during expiration. In contrast to experiments on perfused guinea-pig's lungs there is little leakage from the surface, but the accumulated fluid is easily squeezed out from the cut veins at the hilus by strong over-ventilation for a minute or two. If normal ventilation is restored the lungs again become swollen and fluid can be squeezed out by over-ventilation. The fluid is turbid, contains coagulable protein, histamine and a substance (or substances) which causes a slow contraction of the isolated jejunum of the guinea-pig and temporary changes in the reactivity of the muscle to histamine.

Figs. 2 and 3 illustrate the output of histamine and of coagulable protein in a typical experiment. The upper and lower lobes of the left lung from a 7·7 kg. dog were perfused. These weighed about 25 g. The right lung contained 40 μg. of histamine per g. which indicated a content of about 1 mg. histamine for the perfused lobes. Perfusion at a rate of 8–10 c.c. per min. was continued for 40 min. before the injection of 12 mg. of cobra venom in 1·5 c.c. was made. The perfusate was tested in Fig. 2 on the guinea-pig's jejunum. The perfusate (0·4 c.c.) collected before the injection of venom was without effect (at B). At C to H, 0·2 c.c. of the successive samples after the injection were tested. That tested at C was collected before squeezing out the fluid from the lung by over-ventilation.

Fig. 3 gives the output in μg. per min.; it reached its maximum in the second half hour after the injection, 454 μg. being assayed in this sample. The total output during 2½ hr. was 942 μg. and another 4 μg. were found in the fluid draining from the lung after the perfusion was stopped ("drainage fluid"). The tissue should now have been practically depleted of its histamine and this was found to be the case. The lung, after drainage, weighing 44 g. contained 22 μg. of histamine.

Fig. 2.

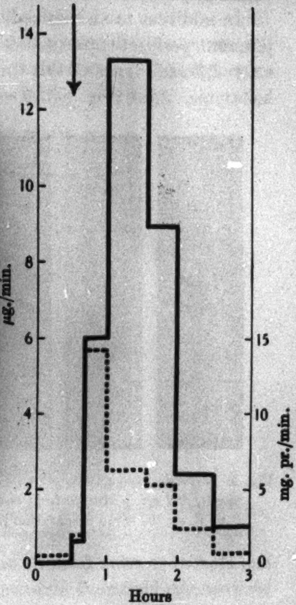

Fig. 3.

Fig. 2. Record from guinea-pig's jejunum desensitized to cobra venom. At A and I, 0·04 μg. histamine; at B to H, boiled perfusate from dog's lung. At the arrows, bath washed out. (Details in text.)

Fig. 3. Output of histamine (continuous line) and of protein (dotted line) from perfused dog's lung. At the arrow 12 mg. cobra venom. Ordinates on the left, histamine in μg. per min. and on the right, protein in mg. per min. Abscissae, time in hours.

The concentration of coagulable protein was strongest in the samples collected 10–30 min. after the injection of the venom, the maximum output preceding that of histamine (Fig. 3). In experiments on the effect of bee venom on the perfused lung of the dog we found [1937*b*] a parallelism between the histamine and protein concentration of the different samples of perfusate. In the present experiment the rate of perfusion was greater, which may account for the difference in the results.

In addition to the immediate rapid contraction of the guinea-pig's jejunum, perfusate often caused a delayed slow contraction. Figs. 4 and 5 show different types of this contraction complicating the rapid effect of histamine. At A (Fig. 4) the secondary contraction set in before the gut had started to relax from its histamine contraction; at B the distinction between the histamine and secondary contractions is better defined. At C and D after washing out the perfusate the secondary contraction set in before the muscle had relaxed completely, and in Fig. 5 it appeared merely to delay the normal relaxation of the muscle. Further variations in the response of the muscle are shown in Fig. 8 for perfusate collected from a monkey's lung. The different types of contraction are determined by the sensitivity of the preparation to histamine and to the substance causing the slow contraction, by the concentrations of both substances in the samples tested and by the length of time allowed in contact with the muscle. When a sample of perfusate is brought to the boiling point and then cooled, its action in causing the secondary contraction is somewhat reduced. In Fig. 4 are shown the effects of a sample of perfusate before (at C) and after boiling (at D). When perfusate is repeatedly tested (ten

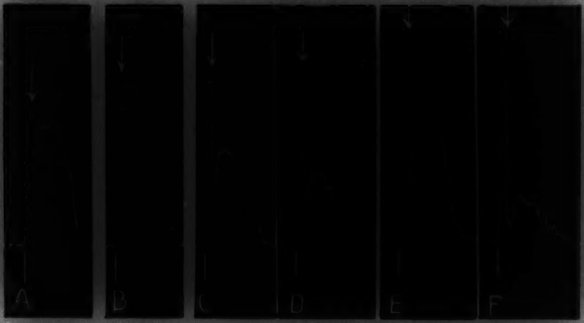

Fig. 4. Records from guinea-pig's jejunum desensitized to cobra venom (three preparations). 0·2 c.c. perfusate from envenomed dog's lungs (A to D) and liver (E and F). C to F, on the same preparation (D and F boiled). Time in 30 sec. (Details in text.)

to twenty times) on the same preparation and particularly when large amounts of perfusate are used, the muscle may gradually become less sensitive to the slowly contracting principle although its response to histamine is not weakened. In this condition the preparation is best suited for the assay of histamine in the perfusate. In Fig. 2, for instance, the assay was carried out on a preparation which had been used for several hours and had become somewhat insensitive to the slowly contracting substance. Furthermore, the samples of perfusate were boiled before testing and left in contact with the muscle for 5–7 sec. only.

Fig. 5. Record from guinea-pig's jejunum desensitized to cobra venom. At A, 0·2 c.c. perfusate from envenomed dog's lung. The remaining responses are to 0·04 μg. histamine. Time in 30 sec.

When the muscle has relaxed from the secondary contraction it is sometimes, at first, more, and later, less sensitive to histamine. The former state lasts only a few minutes, the latter for times up to 20 min. This is illustrated in Fig. 5. Frequently only one of these states is observed.

The principle causing the slow contraction and the changes in excitability of the muscle is formed by the action of the venom. This becomes apparent when the effects on the guinea-pig's jejunum of extracts of normal and envenomed lungs are compared. Normal lung usually causes only a histamine effect though there may be a slight after-contraction; with envenomed lung the histamine effect is followed by a pronounced after-contraction. In Fig. 6, A gives the effect of boiled saline extract of 5 mg. of normal lung, and B that of 10 mg. from the same lung after injection of 20 mg. of cobra venom and 90 min. perfusion. Since the lung weight had doubled the doses correspond. It will be seen, at A, that the muscle, which had begun to relax while the extract was present in the bath, rapidly reached its original length when the Tyrode solution was changed after 30 sec. At B, the initial histamine contraction was weaker than that at A, part of the lung histamine having been liberated, but there was an after-contraction from which the muscle recovered gradually when the Tyrode solution was changed. The after-contraction caused by envenomed lung was followed by changes in excitability of the muscle similar to those observed after venous perfusate. In Fig. 6 there was a period of decreased reactivity to histamine.

Fig. 6. Record from guinea-pig's jejunum desensitized to 100 μg. cobra venom. At A, extract of normal, at B, of envenomed dog's lung. The remaining responses are to 0·04 μg. histamine. At the arrows, bath washed out. Time in 30 sec. (Details in text.)

C. *Monkey's lungs*

We have perfused the left lungs of two monkeys; the results were essentially the same as those described for the dog's lung. The lungs weighed about 15 g. and contained 80 and 100 μg. of histamine per g. respectively. Before the injection of venom the perfusate contained no detectable amount of histamine. After the injection the respiratory movements diminished, the outflow became slower without diminution of the inflow and the lung became swollen and glassy in appearance. The accumulated fluid could be squeezed out by over-ventilation. Histamine, protein and the substance (or substances) which caused slow contraction and changes of excitability of the isolated guinea-pig's jejunum appeared in the perfusate.

Fig. 7 illustrates the histamine and protein output in one of these experiments after the injection of 10 mg. of cobra venom. The histamine output rose to 11 μg. per min. in the first ½ hr. and then fell again rapidly. The total output was 872 μg. and 25 μg. were assayed in the "drainage fluid". This corresponded to 87 % of the histamine available in the perfused lung. In the other experiment 2 mg. of cobra venom in 1 c.c. of saline

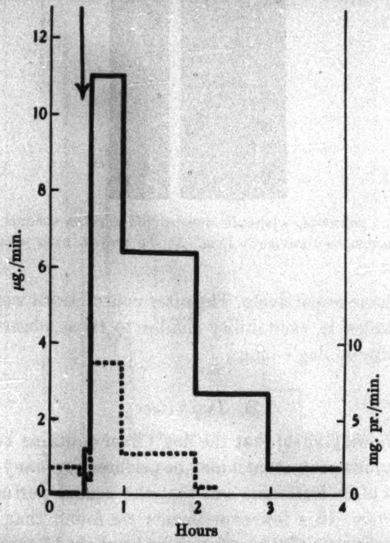

Fig. 7. Output of histamine (continuous line) and of protein (dotted line) from perfused monkey's lung. At the arrow, 10 mg. cobra venom (in 1 c.c.). Ordinates and abscissae as in Fig. 3.

solution were injected. The histamine output was 698 μg. during 2½ hr. perfusion and 40 μg. were found in the "drainage fluid". The total output of 738 μg. amounted to 53 % of the lung histamine, the lung after drainage yielding 664 μg. by extraction.

The substance causing after-contraction appeared to be present in greater amount than in the perfusate from envenomed dog's lungs; it was most concentrated in perfusate collected during the first 1 or 2 hr. after the injection of venom. In Fig. 8 are shown the effects on the gut of perfusate from the experiments of Fig. 7. At A, 0·04 c.c. of the sample collected 35–95 min., and at B, 0·2 c.c. of that collected during the last hour of perfusion, were added to the bath. The immediate sharp contractions were due to histamine present in concentrations of about 1 : 1 million and 1 : 10 millions respectively. The after-contractions were followed by evanescent changes in excitability similar to those observed in experiments with perfused dog's lungs.

Fig. 8. Record from guinea-pig's jejunum desensitized to cobra venom. At A and B, perfusate from envenomed monkey's lung. At the arrows, bath washed out. (Details in text.)

D. *Dog's liver*

We have shown [1937*b*] that the dog's liver contains between 20 and 110μg. of histamine per g.; that it may be perfused for many hours without detectable loss of its histamine and that the venous perfusate is usually devoid of activity. In a few experiments we found that perfusate collected at the beginning of the perfusion had a slight histamine-like action on the gut. When perfusion was continued for 1 or 2 hr. this activity diminished and eventually disappeared.

Intraportal injection of a single large dose of venom (5 *mg. or more*) *into the perfused liver*. After the injection the liver becomes somewhat swollen and loses its brownish colour. Depigmentation seems to affect first the region of the lobule near Glisson's capsule giving the liver a reticulated appearance, but gradually the whole lobule is affected. If the venom is evenly injected all parts of the organ participate in this change, otherwise those which are not reached by the venom retain their brown colour. The venous perfusate assumes a brownish yellow tint. This is deepest in the samples collected during the first few minutes after the injection; 20–50 min. later the brownish yellow colour has usually disappeared from the perfusate. When the blood has not been completely washed out from the liver, the early samples collected after the injection contain blood pigments. The perfusate, after the injection becomes turbid, contains protein, histamine and the substance (or substances) which causes slow contraction and the changes in reactivity of the gut.

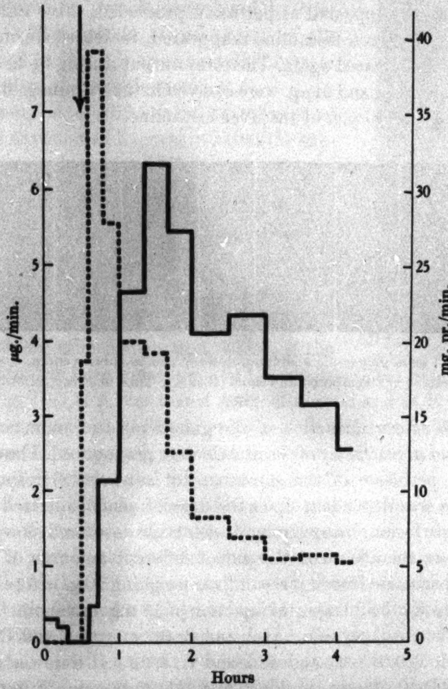

Fig. 9. Output of histamine (continuous line) and of protein (dotted line) from perfused dog's liver. At the arrow, 7·5 mg. cobra venom. Ordinates and abscissae as in Fig. 3.

In Fig. 9 is shown the output of protein, and of histamine from a piece of liver weighing 60 g. The output of protein reaches its maximum in the second sample of perfusate collected 5–15 min. after the injection amounting to 390 mg.; the total output during 220 min. perfusion was 2·8 g. The maximum output of protein precedes that of histamine, but follows that of liver pigment. The loss of protein from the liver was apparent when saline extracts of perfused normal and envenomed liver were boiled. The protein in the former was coagulated in large flakes and in the latter in fine flakes.

The liver from the experiment of Fig. 9 contained 36μg. of histamine per g. The perfusate, before the injection, had some histamine-like activity, which disappeared as perfusion proceeded. Five minutes after the injection of venom histamine reappeared, increased during the first hour and then decreased again. The total output during 3½ hr. perfusion amounted to 822μg., and 24μg. were assayed in the "drainage fluid". This represented about 40 p.c. of the liver histamine.

Fig. 10. Record from guinea-pig's jejunum desensitized to cobra venom. Perfusate from dog's liver before (A) and after cobra venom (B to K). Time in 30 sec. (Details in text.)

The slow after-contraction of the guinea-pig's jejunum and the ensuing changes in reactivity of the muscle were pronounced. The concentration in the perfusate of the substance (or substances) responsible for these effects was dependent upon the dose of venom injected and upon the rate of perfusion, being stronger with large doses and slow perfusion. Fig. 10 shows the effects on the gut of different amounts of successive samples of perfusate from a piece of liver weighing 70 g. before (at A) and after (at B to K) the intraportal injection of 18 mg. of venom. The rate of perfusion was 1 c.c. per min. At A and B, 0·2 c.c., at C and D, 0·04 c.c., at E, F and G, 0·01 c.c., and at J and K, 0·02 c.c. were tested. B was from a sample 10–20 min., C, 20–30 min., D, 20–60 min., E in the second, F in the third, G in the fourth, J in the fifth and K in the sixth hour after the injection. The concentration of the slowly contracting substance first increased and then decreased again following roughly the same course as the output of histamine. The perfusate after boiling lost much of its action in causing slow contraction, whereas the histamine-like activity remained unimpaired. Boiling had more effect upon perfusates from envenomed liver than on those from envenomed lungs. This is illustrated in

Fig. 4, which shows the effects of unboiled (C and E) and boiled (D and F) perfusates from envenomed lungs and liver of a dog. In spite of this difference in behaviour the after-contraction is probably caused by the same substance in both perfusates. Perfusate from the liver contains more protein than that from the lung and differences in the reduction of activity by boiling may result from adsorption to protein coagulated by heat (*vide infra*). The protein concentration of the sample tested at Fig. 4E was about five times as great as that of the sample tested at C.

Fig. 11. Record from guinea-pig's jejunum desensitized to cobra venom. Boiled saline extract of normal (A) and envenomed dog's liver (B). Time in 30 sec. (Details in text.)

A comparison of the effects of extracts of normal and envenomed livers shows that the substance causing the slow contraction must be formed in the organ by the action of the venom. Saline extracts of a few milligrammes of normal liver usually cause only an immediate histamine effect, although there may in some instances be a slight after-contraction. With extracts from envenomed liver the histamine effect is weaker, but there is a strong after-contraction, which, if the histamine loss has been great, may be the sole response. Such a result is shown in Fig. 11. At A was tested 2 mg. of normal and at B 2 mg. from the same liver extracted 90 min. after the injection of venom. The contraction at A started immediately; that at B commenced after a latent period of 18 sec. and developed and relaxed more slowly.

Repeated intraportal injections of small doses of venom into the perfused liver. With smaller doses of venom the depigmentation of the liver and the output of protein, of histamine and of the substance causing the slow contraction were less extensive. In one experiment, in which 1 mg. of cobra venom (in 0·2 c.c.) was injected into a piece of liver weighing 77 g., 22% of the liver histamine was recovered in the perfusate during 6 hr. In another, in which 51 g. of liver containing about 4·5 mg. of histamine were perfused, the injection of 0·2 mg. of cobra venom (in 0·2 c.c.) produced an output of only 40μg. of histamine. Doses of venom of this order in a larger volume, if injected for the first time, usually caused no output of histamine. But if the injection was repeated, histamine appeared in the perfusate in increasing amounts with successive injections (Fig. 12). A piece of liver weighing 70 g. and containing 36μg. of histamine per g. was perfused and four successive injections of 0·3 mg. of venom (in 1·5 c.c.) were made. In the perfusate collected after the first injection (at A) there was no detectable amount of histamine, though it contained protein and in the sample collected during the first 5 min. some pigment was present. This sample if added to the isolated jejunum in amounts of 0·4 c.c. caused a slight slow contraction after a latent period of some 20 min. The second injection of venom given 30 min. later (at B) caused an evanescent output of 1·8μg. of histamine. The output of protein was increased and the sample collected during the first 5 min. had a brownish tint and contained small amounts of the principle that caused slow contraction of the gut. The histamine output after the third injection (at C) amounted to 5·8μg. and was more delayed; it had not come to an end after 30 min., when the fourth dose of venom was injected. This caused an immediate output of histamine followed by a long-lasting secondary output, which was still increasing after 2¼ hr. when perfusion was stopped. The total histamine assayed during this period amounted to 111μg.

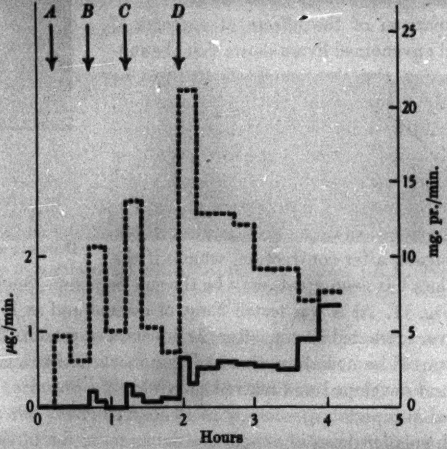

Fig. 12. Output of histamine (continuous line) and of protein (dotted line) from perfused dog's liver. At the arrow, 0·3 mg. cobra venom. Ordinates and abscissae as in Fig. 3.

Perfusion of the liver following on intravenous injection of venom. In a few experiments we have tried to ascertain whether the liver of a dog poisoned by intravenous injection of cobra venom would give off its histamine during an ensuing perfusion. A dose of 2 mg. per kg. of venom was injected intravenously into dogs under chloralose anaesthesia. 30–60 min. later, when the animals were about to die, a cannula was tied into the portal vein and perfusion of the swollen liver commenced. The perfusate collected during the first hour was very turbid and reddish, containing protein, blood corpuscles and blood pigment. Samples tested on the gut caused a strong slow contraction but there was no indication of the presence of histamine. Later on the protein content and the reddish colour diminished and histamine appeared in increasing amounts, the histamine effect being followed by strong after-contraction. The liver gradually became somewhat depigmented, but even after several hours it showed reddish areas on section and the perfusate still contained small amounts of blood pigment. In Fig. 13 is shown the output, from the first hour onward, of histamine and of protein from a piece of liver weighing 139 g. at the end of the perfusion. Perfusion of the liver was started 30 min. after the injection of 20 mg. of cobra venom into a dog weighing 10 kg. The output of histamine during 5 hr. amounted to nearly 1 mg. which corresponded to about 20% of the available histamine. The output of protein was large, and early in the second hour it amounted to 60 mg. per min. Probably part of it was derived from plasma and blood corpuscles accumulated in the tissue spaces of the swollen liver.

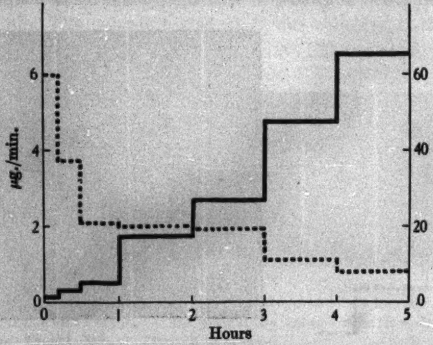

Fig. 13. Output of histamine (continuous line) and of protein (dotted line) from perfused liver of envenomed dog. Ordinates and abscissae as in Fig. 3. (Details in text.)

E. *Monkey's liver*

The histamine equivalent per g. of liver is less than 1 μg. and generally less than 0·5 μg. After the intraportal injection of cobra venom (10 mg. or more) into the perfused liver, depigmentation occurs. If the venom is evenly injected all parts of the liver participate, otherwise those which are not reached by the venom retain their brown colour. Liver pigments appear in the perfusate, reaching their maximum concentration during the first 10–15 min. after the injection. There is little swelling of the organ, the edges of which remain sharply defined, but the perfusate becomes cloudy and contains protein. Fig. 14 shows the output of protein per min. from a piece of liver weighing 73 g. after the injection of 10 mg. of cobra venom. The total output of protein was 1·6 g.

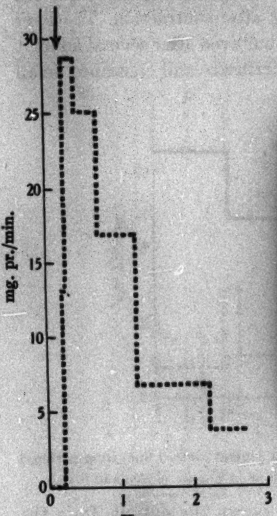

Fig. 14.

Fig. 15.

Fig. 14. Output of protein from perfused monkey's liver. At the arrow, cobra venom. Ordinates, mg. of protein per min. Abscissae, time in hours.

Fig. 15. Record from guinea-pig's jejunum desensitized to 100μg. cobra venom. At A, B and C, unboiled perfusate; at D and E, unboiled saline extracts from monkey's liver, at H, 0·04μg. histamine. Time in 30 sec. (Details in text.)

No immediate histamine-like contraction could be elicited on the guinea-pig's jejunum with perfusate before or after the injection of venom. Samples collected before had no stimulating action, and those collected after caused only delayed slow contraction. This is shown in Figs. 15 and 16. In Fig. 15, 0·2 c.c. of perfusate collected before (at A) and in the second ½ hr. after the injection of 30 mg. of cobra venom (at B), were tested and kept in contact with the muscle for 30 sec. The rate of perfusion was 7 c.c. per min. The contraction at B commenced after a latent period of 10 sec. and was less rapid than that caused by histamine. At C, 0·02 c.c. of this sample caused a slow contraction after a latent period of 20 sec. In some experiments 0·002 c.c. of perfusate was active. If the perfusion of the liver was continued for a longer period, the activity of the perfusate gradually decreased. In Fig. 16 are shown the effects on the gut of 0·2 c.c. of perfusate collected before (at A) and in successive samples after the injection of venom, the rate of perfusion being between 6 and 7 c.c. per min. The sample tested at B was collected 5–15; that at C, 15–30; at D, 30–60; at E, 60–120; at F, 120–150 and at G, 150–170 min. after the injection. The samples from B to G diminished progressively in activity. There was no change in the sensitivity of the gut, which contracted strongly when the sample tested at B was retested at J. The latent period lengthened with the successive samples being less than 20 sec. at B and more than 30 sec. at G. Perfusate after boiling was less active in causing slow contraction of the gut. In Fig. 19 the effects of 0·2 c.c. of a sample of perfusate before (at A) and after boiling (at B) are compared.

Fig. 16. Record from guinea-pig's jejunum desensitized to cobra venom. Boiled perfusate from monkey's liver before (A) and after 10 mg. cobra venom (B to J). At H, 0·04 μg. histamine. Time in 30 sec. (Details in text.)

After recovery from the slow contraction the reactivity of the muscle to histamine was altered in the manner already described. The response to other stimulating substances, e.g. acetylcholine, was similarly affected. When the muscle exhibited spontaneous rhythm this was often increased during increased excitability and disappeared during the period of depressed excitability, during which the muscle was in a slightly more relaxed condition. Depressed excitability is illustrated in Fig. 19 A.

The substances causing slow contraction and changes in excitability of the gut must be formed by the action of the venom. A similar conclusion was reached from the experiments on dog's organs. Saline extracts of normal monkey's liver in amounts corresponding to 1–2 mg. of tissue have no action on the gut, but in doses of 5 mg. or more cause delayed slow contraction followed by the typical changes in reactivity. This observation indicates the presence in normal liver of small quantities of a substance, which may be identical with that found in the perfusate after the injection of venom. Extracts of envenomed liver, however, were found to be 20–80 times more active, a slow contraction being produced by amounts corresponding to 0·2–0·5 mg. of liver. In Fig. 15 are seen the effects of 5 mg. of normal (at D) and of 0·5 mg. of envenomed liver (at E). Further evidence for the assumption that the substance is formed in the tissue is given by those experiments in which the venom was not uniformly distributed throughout the organ. Extracts made from the depigmented portions were many times more active than those from regions which had not lost their pigment. Boiling reduces the activity of saline extracts to some extent, but the difference between normal and envenomed liver is still evident. The principle responsible for the slow contraction of the gut is soluble in absolute methyl alcohol, and alcoholic extracts of normal and envenomed liver exhibit similar differences in activity. In Fig. 17 alcoholic extract corresponding to 10 mg. of normal liver was without effect on the gut (at B). The histamine equivalent of this liver was less than 0·5μg. per g., but 40 mg. of liver (at A) caused an immediate histamine effect, followed by slow contraction after changing the Tyrode solution. Extracts of envenomed liver (at C, D and E) were effective in much smaller amounts.

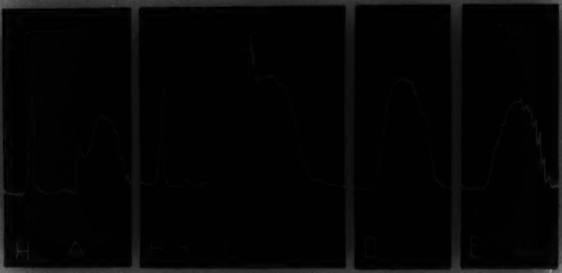

Fig. 17. Record from guinea-pig's jejunum desensitized to cobra venom. At A, 40 mg. and at B, 10 mg. normal monkey's liver extract. At C, 5 mg., at D, 1 mg. and at E, 0·5 mg. "envenomed liver" extract. At H, 0·04μg. histamine. Time in 30 sec.

PART 2. COMPARISON OF THE ACTIONS OF "ENVENOMED LIVER", "LYSOCITHIN" AND COBRA VENOM

The fact that extracts from envenomed liver of the monkey yielded a rich supply of the substance which causes the slow contraction of the guinea-pig's gut enabled us to use them as a source of the substance for pharmacological investigation. In preliminary experiments saline extracts were used, which were boiled and kept in the cold room for a day or two without loss of activity. The experiments recorded here were made with extracts of monkey's liver in absolute methyl alcohol in which the active principle, like lysocithin, is soluble and stable.

To test the hypothesis that lysocithin-like substances are responsible for the activity of perfusate and of extracts of envenomed liver we had first to ascertain whether the extract made by the action of cobra venom on lecithin ("lysocithin") caused similar effects on the jejunum of the guinea-pig and whether extracts of envenomed liver resembled lysocithin in haemolytic activity. We shall see that "lysocithin" and "envenomed liver" possess both these actions. These may be properties of a single substance, but they may equally well be properties of different substances which may be derivatives of lecithin or of other lipins present as impurities. Sodium oleate had no stimulating action on the guinea-pig's gut, even in amounts much greater than could have been present in our extracts of lysocithin. This excludes the possibility that oleic acid itself is responsible.

The possibility that the formation of lysocithin-like substances might be an intermediate stage in the liberation of histamine made it necessary to ascertain whether "lysocithin" itself and extracts of envenomed liver could cause the liberation of histamine from perfused organs. If these hypotheses were substantiated, the formation of lysocithin-like substances must play a significant role in poisoning by cobra venom. We have, therefore, compared some additional actions of "envenomed liver", of "lysocithin" and of cobra venom, including some which cannot be attributed to histamine.

A. *Guinea-pig's jejunum*

The actions of "envenomed liver", "lysocithin" and cobra venom on the guinea-pig's jejunum are similar. All three cause a slow contraction after a latent period of 5–30 sec., and when the muscle has recovered it may exhibit periods of increased and decreased reactivity to histamine and other stimulant substances (Fig. 19). Whereas the muscle is readily desensitized to cobra venom by its repeated administration, such a phenomenon is not characteristic for "envenomed liver" or "lysocithin".

"*Envenomed liver.*" Fig. 17 illustrates slow contractions caused by different amounts of extracts. These contractions are usually followed by the characteristic changes in reactivity of the muscle to histamine and acetylcholine. Fig. 18 illustrates increased reactivity to histamine following the contraction caused by 1 mg. of "envenomed liver" (at A), and Fig. 19 a long-lasting period of decreased reactivity following the contraction caused by 10 mg. (at C). These results are unaffected by desensitization of the gut to cobra venom. When "envenomed liver" is given repeatedly the contractions gradually become weaker, and in some instances the muscle becomes almost insensitive to it though not to histamine. In a few experiments this condition was precipitated by cobra venom (10–40μg.) given after several doses of "envenomed liver". A similar condition was produced by repeated administration of large amounts of alcoholic extracts of normal liver (50–100 mg.).

Fig. 18. Record from guinea-pig's jejunum desensitized to cobra venom. At A, 1 mg. "envenomed liver"; at B, "lysocithin" equivalent to 150μg. lecithin. The remaining responses are to 0·04μg. histamine. Time in 30 sec.

In contrast to the behaviour of perfusates from "envenomed liver" watery solutions of the alcoholic extract do not lose activity by boiling. Nevertheless, the principle causing slow contraction in perfusates and extracts appears to be the same, since extract added to perfusate becomes less active by boiling. To two samples of 10 c.c. of the same perfusate from envenomed liver the same amount of extract was added, in one before, and in the other after, boiling. The latter mixture was more active.

The slow delayed contraction caused by perfusate or extract from envenomed liver, though closely resembling the response of the gut to cobra venom, cannot be attributed to the presence of the venom. The

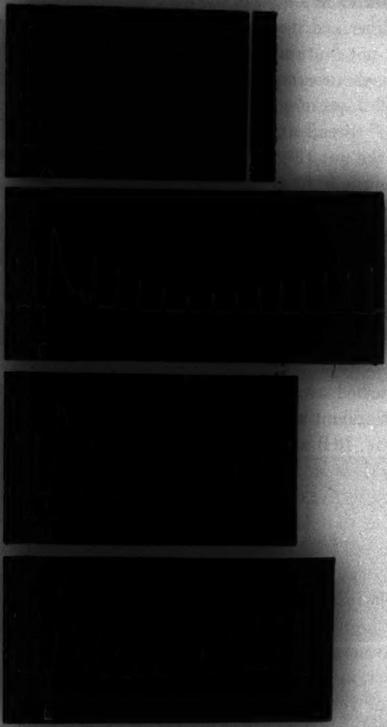

Fig. 19. Records from jejunum from three different guinea-pigs; A and B on one preparation. A and B, perfusate (0·2 c.c.) from envenomed monkey's liver; A, unboiled; B, boiled. At C, 10 mg. "envenomed liver". D and E on one preparation; at D, "lysocithin" equivalent to 200μg. lecithin; at E, 30μg. cobra venom. Doses left in contact for 30 sec. The remaining responses are to 0·04μg. histamine. Time in 30 sec.

contractions were usually produced on a gut desensitized to 50 or 100μg. of venom, so that to be effective venom must have been present in amounts larger than these. This was not possible. The contractions shown in Fig. 16 were produced by different samples of 0·2 c.c. of perfusate the total amount of which exceeded 1 litre. Since in this experiment 10 mg. of venom had been injected the amount present in 0·2 c.c. must have been less than 2 μg. The alcoholic extract of the 60 g. of liver at the end of the perfusion was active in a dose of 0·5 mg., and even if all the venom were present in the liver and it were soluble in alcohol, which is not the case, this dose could not contain more than 0·1 μg. Finally, repeated administration of perfusate or extract from envenomed liver did not desensitize the gut to small doses of cobra venom.

"*Lysocithin.*" Small amounts of "lysocithin" cause a delayed slow contraction of the gut (Figs. 18 B, 19 D and 20). The sensitivity of preparations of jejunum from different guinea-pigs varied greatly, independently of their reactivity to histamine. On some, "lysocithin" equivalent to 4 μg. of lecithin caused a strong reaction, on others equally sensitive to histamine 10–30 times this dose was required for a similar response. There was usually a close resemblance between these effects and those produced by "envenomed liver" on the same preparation, though sometimes relaxation of the muscle was slower after "envenomed liver" than after "lysocithin". The muscle relaxed gradually when the "lysocithin" was washed away and exhibited the characteristic changes in reactivity to stimulant substances. Increased reactivity to histamine is illustrated in Fig. 18 B and decreased reactivity in Fig. 19 D. The "lysocithin" contractions in Figs. 18 and 19 started after a latent period of 15 sec. In Fig. 18 the change in excitability is compared on the same preparation with that following a corresponding contraction caused by "envenomed liver" (at A) and in Fig. 19 with that following a contraction by cobra venom (at E). In those preparations in which the contraction caused by "lysocithin" was not followed by changes in reactivity of the muscle to histamine, such changes were absent after the contraction caused by "envenomed liver" or by cobra venom.

The contraction caused by "lysocithin" closely resembles that produced by cobra venom, but the method, by which "lysocithin" was prepared, excluded the presence of venom in the final methyl alcohol extract and the contraction was usually not influenced by desensitization to cobra venom (Fig. 20). At A, D and E the same amounts of "lysocithin" were added to the bath. B shows the effect of 10 μg. of cobra venom, a second dose of venom given at C was ineffective, but the muscle still reacted to "lysocithin". Between D and E three doses of 100 μg. of cobra venom were given, the first and second caused slow and weak contractions, the third had no effect. Though the muscle was desensitized to doses of venom of this order, its reactivity to "lysocithin" was not materially altered.

The effect of "lysocithin" could be obtained repeatedly on the same preparation but the contractions gradually became weaker and sometimes were further reduced by cobra venom. The reaction to histamine under these conditions remained unchanged or increased. We have described the same course of events after repeated doses of "envenomed liver".

Lysocithin is resistant to heat and the preparation of our alcoholic extracts involved heating for considerable periods. Boiling a watery solution of "lysocithin" did not diminish its activity, but when it was added to perfusate from envenomed monkey's liver and brought to the boiling point its activity was reduced like that of "envenomed liver".

In a few experiments we have examined the influence of alcoholic extract from normal monkey's liver on the action of "lysocithin". Alcoholic extracts of "lysocithin" and of normal liver were evaporated together and taken up in Tyrode solution. It was found that extracts of a few milligrammes of normal liver did not diminish the contraction caused by "lysocithin", though a slightly longer latent period suggested some inhibitory influence.

Fig. 20.

Fig. 21.

Fig. 20. Record from guinea-pig's jejunum. At A, D and E, "lysocithin"; at B and C, 10 μg. cobra venom. At H, 0·04 μg. histamine. (Details in text.)

Fig. 21. Record from guinea-pig's jejunum. At A, third administration of 10 μg. cobra venom. The remaining responses are to 0·02 μg. acetylcholine. Time in 30 sec.

Cobra venom. In addition to its stimulating action venom causes the same changes in reactivity as described for "envenomed liver" and for "lysocithin". The strong contraction of a first dose of venom is often followed by decreased reactivity, whereas a slight stimulating effect produced on the partly desensitized muscle by the same dose of venom applied for the second or third time is followed by increased reactivity, this dose of venom produces no changes of reactivity when the muscle is completely desensitized. This indicates that the changed reactivity results from an indirect action of the venom and it is reasonable to assume that the formation of lysocithin-like substances in the muscle is responsible.

Fig. 19 illustrates decreased reactivity to histamine after a contraction caused by 30μg. of venom (at E). The muscle had previously been subjected to 10μg. of venom but was only partly desensitized. The contraction started after a latent period of 12 sec. The muscle exhibited small spontaneous movements which disappeared during the period of decreased reactivity. In Fig. 21 is shown the effect of a third dose of 10μg. of cobra venom on another preparation (at A). The weak contraction is followed by a short period of increased reactivity to acetylcholine.

B. *Guinea-pig's uterus*

The uterus of the guinea-pig responds by contraction to cobra venom but is easily desensitized. "Lysocithin" and the active substances in perfusate and extract of envenomed liver also cause contraction which is not affected by previous desensitization with venom and can be repeatedly elicited without appreciable diminution. Fig. 22C illustrates a contraction produced (at I) by "lysocithin" equivalent to 2 mg. of lecithin and Fig. 22D a contraction (at I) caused by 10 mg. of "envenomed liver".

C. *Guinea-pig's uterus poisoned by histamine*

Schild [1936] has shown that the uterus poisoned by large doses of histamine responds to the addition of further histamine by relaxation but that the anaphylactic contraction can still be elicited. He concludes "that either the histamine released from the cells has a different action from that of histamine applied to the cell outer surface or it plays only a secondary part in anaphylactic shock".

The uterus poisoned by histamine still contracts to cobra venom "lysocithin" and "envenomed liver" though the contractions have a longer latent period and are more gradual and less in extent than those of the normal uterus. Fig. 22B illustrates the slow contraction (at I) of the poisoned uterus to a first dose of 20μg. of cobra venom. The contraction caused by such a dose on a normal uterus is stronger and quicker. Fig. 22C shows that the "lysocithin" contraction of the poisoned uterus (II) is slower and weaker than that caused on the normal uterus (at I). The latent period at I was 4 sec., that at II, 30 sec. At II in Fig. 22D is shown the contraction of the poisoned uterus caused by 40 mg. of "envenomed liver". It started after a latent period of 18 sec. The contraction of the normal uterus (at I) was caused by 10 mg. of "envenomed liver" and commenced after a latent period of 8 sec.

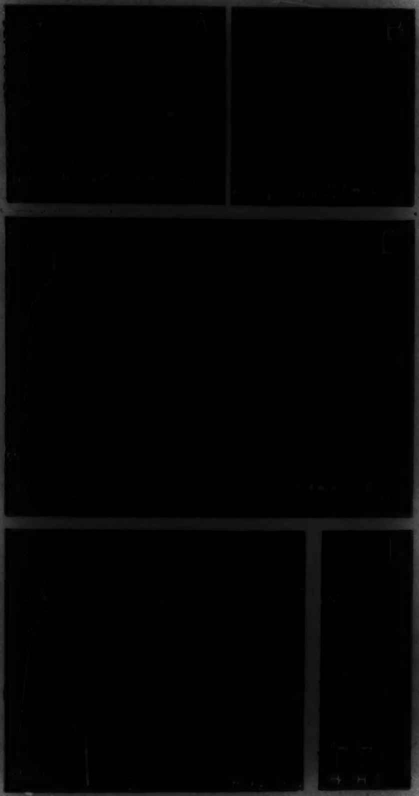

Fig. 22. Records from the uteri of four virgin guinea-pigs. A, at I and II, 10 mg. peptone (Berner). B, at I, 20 μg. cobra venom. C, at I and II, "lysocithin". D, at I and II, "envenomed liver". At H, histamine in various amounts. At the arrows, the bath washed out. Time in 30 sec. (Details in text.)

It was of interest to extend these observations to another cell-injurious agent—peptone, which like "lysocithin" and "envenomed liver" has the advantage that several doses can be given to the normal uterus without appreciable reduction in the extent of the contraction and that comparison of the effects before and after poisoning can be made on the same horn of the uterus. Such an experiment is illustrated in Fig. 22 A, which shows the effect of 10 mg. of peptone before (at I) and during histamine poisoning (at II). The contraction at II is weaker, more gradual and starts after a longer latent interval than that at I.

D. *Rat jejunum*

The jejunum of the rat is insensitive to histamine but responds by contraction to concentrations of 1 : 40,000 to 1 : 50,000. It also contracts with cobra venom, "envenomed liver" and "lysocithin", but individual variation in sensitivity is great and a few preparations did not respond to any of the three substances though they reacted well to acetylcholine. The gut is readily desensitized to cobra venom, but this has little effect on its reaction to "envenomed liver" or "lysocithin". By repeated administration of "envenomed liver" or of "lysocithin" the contractions usually diminish, and sometimes fail to occur. Fig. 23 shows the effect of two successive doses of 10μg. of cobra venom (at A and B) and on another gut of 5 mg. of "envenomed liver" (at C). The contractions start after a latent period of about 20 sec., the muscle continues to contract after changing the Tyrode solution and only relaxes slowly.

Fig. 23. Records from jejunum of two rats. At A and B, 10μg. cobra venom on same gut; at C, 5 mg. "envenomed liver". At Ac, 0·08μg acetylcholine. At the arrows, bath washed out. Time in 30 sec.

E. *Cat's heart*

"Envenomed liver" in contrast to "lysocithin" has a relatively feeble action on the cat's heart. This discrepancy may be accounted for by the presence in liver extracts of substances which antagonize the toxic action of "lysocithin" on the heart. The action of "lysocithin" is not wholly comparable to that of cobra venom.

"*Envenomed liver.*" Filtered extracts cause acute changes in the coronary flow and have a more gradual effect upon the heart beat. A first dose causes dilatation of the coronary vessels which lasts from 4 to 7 min., but no dilatation occurs following subsequent doses. In the course of 2 hr. the heart beats decrease in force without significant changes in rate, and a gradual diminution in coronary flow occurs. If the heart does not recover it becomes oedematous and irregularities of conduction ensue. It finally comes to rest in diastole or in the mid-position but not in systole. The left ventricle first ceases to beat, then the right ventricle and finally the auricles. In the experiment of Fig. 24, 1 g. of "envenomed liver" and 1 hr. later 2 g. were injected. The first injection (at I) produced an increase in coronary flow from 50 to 62 c.c. per min.; the flow then decreased continuously. The force of the heart beats diminished gradually but some recovery occurred. The rate diminished only slightly. After the second injection (at II) there was no coronary dilatation, the force of the heart beats decreased and extra-systoles appeared within 40 min. (at *h*) leading to heart block within an hour (at *j*). Eventually (at *k*) the whole heart stopped in diastole for periods of 5–10 sec., between which there were irregular groups of contractions of the right heart. Later on the periods of diastolic standstill became longer. Equivalent amounts of extracts from normal monkey's liver were without effect.

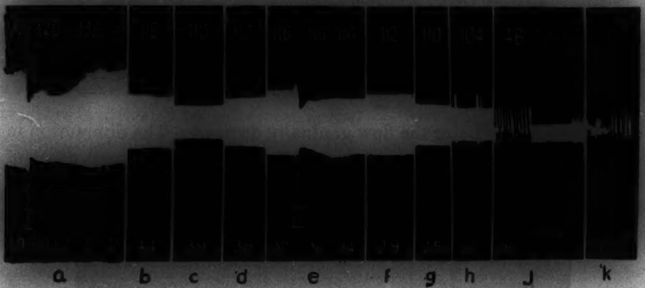

Fig. 24. Record from isolated cat's heart. At I, 1 g., at II, 2 g. "envenomed liver". In this and the following three figures the numbers above are heart rate per min.; below, coronary outflow in c.c. per min. *b* to *e*, 20, 30, 50 and 60 min. after I; *f* to *k*, 15, 30, 40, 60 and 80 min. after II. Time in 30 sec.

"*Lysocithin.*" We used a batch of "lysocithin" which, in a dose equivalent to 1 mg. of lecithin, corresponded in its action on the jejunum of the guinea-pig to 5 mg. of the preparation of "envenomed liver" used in the heart experiments. The effects of "lysocithin" in a dose equivalent to 1 mg. of lecithin are shown in Fig. 25 (at I). There is vasodilatation lasting 6 or 7 min., which is preceded during the first minute by decrease in coronary flow. Later the flow gradually decreases. The diminution in extent of the heart beat proceeds rapidly. If repeated small doses of "lysocithin" are given, the early vasodilatation occurs only after the first injection. Doses of "lysocithin" equivalent to 5–10 mg. of lecithin cause immediate vasoconstriction of the coronary vessels which is not followed by dilatation. The extent of the beat and the coronary flow progressively diminish, the heart rate is slowed, irregularities of conduction occur and eventually the heart ceases to beat in diastole or in the mid-position, but not in systole. The left ventricle stops first, then the right ventricle and finally the auricles.

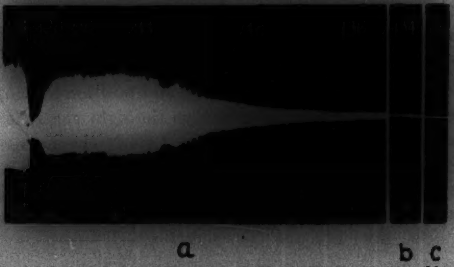

Fig. 25. Record from isolated cat's heart. At I, "lysocithin". *b* is 30, *c* 80 min. after *a*. Time in 30 sec. (Details in text.)

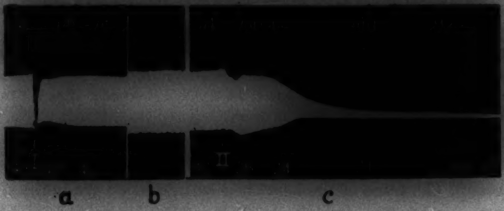

Fig. 26. Record of isolated cat's heart. At I, "lysocithin" with extract of normal liver; at II, "lysocithin" alone. (Details in text.)

In several experiments we have injected different amounts of "lysocithin" with the extract of 1 g. of normal monkey's liver. With 5 and

10 mg. of "lysocithin" the mixtures were without significant effect. If larger amounts of "lysocithin" were injected with this amount of normal liver the changes resembled those produced by the injection of "envenomed liver". The protective action of normal liver extract is illustrated in Fig. 26. "Lysocithin" equivalent to 10 mg. of lecithin, injected at I, together with extract of 1 g. of normal liver produced no obvious changes on the heart beat and only a trivial decrease in coronary flow. Half the amount of "lysocithin" injected alone at II caused rapid failure of the heart, with decrease in coronary flow.

Cobra venom. Unlike many previous workers who have studied the effects of continuous perfusion with various concentrations of cobra venom upon the isolated heart, we have investigated the effects of single injections of venom which pass rapidly through the heart. The injection of 2–4 mg. of venom usually causes systolic contracture of the heart within a few minutes (Fig. 27). The systolic contracture is preceded by a decrease in the extent of the force of the heart beat and by a slight increase in its rate. The coronary flow within a minute after the injection increases nearly twofold and remains rapid though the heart is in systolic contracture. With smaller doses of venom (1 mg.) the changes in the heart beat proceed more gradually. There is diminution in the extent of the heart beat and after some 40–50 min. extra systoles or short periods of heart block occur; but in spite of these the heart is still recording 1½–2 hr. after the injection. If the coronary flow is large (70–80 c.c. per min.) before the injection there is some decrease in flow in the first 1 or 2 min. after the injection; an initial moderate flow is increased by about 50% during the first few minutes. Extracts of normal monkey's liver appear to exert some protective influence against the toxic action of cobra venom.

Fig. 27. Record of isolated cat's heart. At A, 2 mg. cobra venom. Time in 30 sec.

F. *Rabbit's eye*

"Envenomed liver", "lysocithin" and cobra venom when injected into the anterior chamber of the eye cause closely similar and characteristic changes. These have already begun within 4 hr. after the injection and reach their full development in 24 hr. The eye appears to bulge from the socket. The cornea becomes cloudy and so opaque that the pupil can no longer be seen; there is an associated conjunctivitis. Fig. 28 illustrates these changes by photographs: A, 24 hr. after 0·4 g. of "envenomed liver"; B, 24 hr. after "lysocithin" equivalent to 3 mg. of lecithin; and C, 7 days after the injection of 2 mg. of cobra venom. The injections were made in a volume of 0·1 c.c. These changes did not clear up in the course of 2 months. Doses, a tenth to a quarter as large, produced slight and transient congestion of the iris without gross corneal changes. Within 48 hr. the injected eye could not be distinguished from its untreated fellow.

Extract of normal liver in large amounts had no such action. Fig. 28D is a photograph 24 hr. after the admixture of 0·8 g. of normal monkey's liver with the contents of the anterior chamber. The suspended material had been absorbed and the eye was almost normal.

G. *Intravenous injection in guinea-pigs*

The injection of "envenomed liver" and of "lysocithin" into guinea-pigs by a jugular vein, exposed under local anaesthesia, caused similar symptoms starting after a latent interval of 5–15 sec. There was cough and obstructive dyspnoea, and lethal doses caused death from asphyxia. Cyanosis of the ears and convulsions were predominant features, and death was preceded by coma with evacuation of the bladder and the passage of a few faecal pellets. In animals which recovered after severe symptoms, instead of a few terminal and ineffective respirations, the respiratory rate suddenly quickened, the bronchial constriction was overcome, convulsions, if present, ceased, the corneal reflex returned and after a period of collapse the animals were restored almost to normal. These are the symptoms of acute anaphylactic shock in guinea-pigs. In a few animals there was an additional symptom which is never present in acute anaphylaxis—blood-stained frothing from the nostrils, the manifestation during life of acute oedema of the lungs. A similar symptom has been observed by Belfanti [1925] after the intravenous injection of lysocithin into rabbits. There is yet another respect in which the picture differs from acute anaphylaxis. The administration of a sublethal dose of "envenomed liver" or of "lysocithin" fails to protect the animal from a lethal dose of either injected shortly afterwards. In some of the guinea-pigs, in which "envenomed liver" was injected, collapse, lasting 4–10 min., was the dominant feature. Post-mortem the lungs were distended and did not collapse when the thorax was opened, but, on section, oedema and a few haemorrhages were invariably present. The heart was generally beating feebly, the right chambers being full of dark blood. Extracts of normal monkey's liver in dosage equivalent to twice the lethal dose of "envenomed liver" caused no symptoms when injected intravenously.

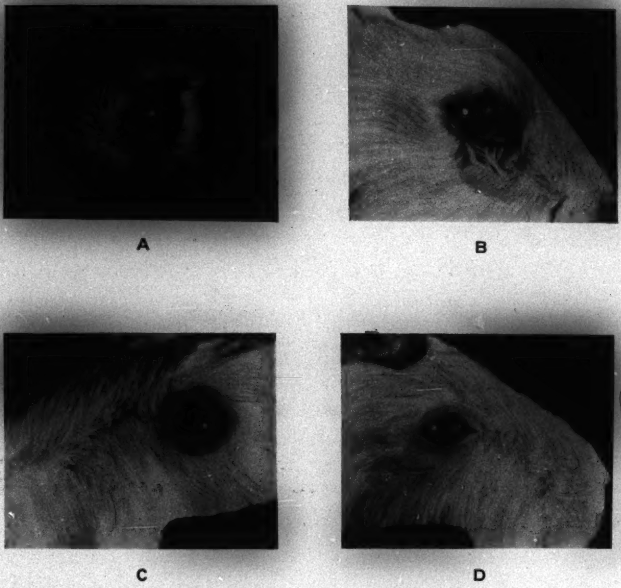

Fig. 28. Photographs of rabbits' eyes after injections into anterior chamber. A, of "envenomed liver"; B, of "lysocithin"; C, of cobra venom, and D, of normal liver extract. (Details in text.)

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The toxicity of "envenomed liver" and that of "lysocithin" were paralleled by their haemolytic action rather than by their stimulant effect upon the isolated jejunum of the guinea-pig. Tables I and II give the results of titrations of the toxicity of an extract of envenomed liver and of a batch of "lysocithin". "Envenomed liver" in a dose of 1 mg. corresponded in toxicity to "lysocithin" equivalent to about 10μg. of lecithin. On washed sheep's corpuscles the haemolytic activity of 1 mg. of this liver extract corresponded to "lysocithin" equivalent to about 15μg. of lecithin.

Table I. Intravenous injection of "envenomed liver" in guinea-pigs

Weight in g.	Dose in g.	Result
280	1·6	+ in 1½ min.
330	1·2	+ in 2½ min.
245	1·2	+ in 2 min.
258	1·2	+ in 1 min. 40 sec.
305	0·8	Severe symptoms during 11 min. Recovery
325	0·8	Severe symptoms during 5 min. Recovery

Table II. Intravenous injection of "lysocithin" in guinea-pigs

Weight in g.	Dose in mg. as lecithin equivalent	Result
250	15	+ in 2½ min.
240	12	+ in 1 min. 55 sec.
270	12	+ in 2¼ min.
300	12	+ in 1 min. 40 sec.
296	9	Severe symptoms for 5 min. Recovery
265	9	Slight symptoms. Recovery
270	7·5	No symptoms

H. *Sheep red blood corpuscle*

"Envenomed liver" has an immediate haemolytic action on washed sheep's corpuscles resembling that of "lysocithin" (Table III). This action is not impaired by boiling (Table IV). Extracts of normal liver and cobra venom are without action on these cells. We tested cobra venom in concentrations up to 20 mg. per c.c.

Table III. Haemolysis times in minutes of washed sheep's red blood corpuscles

		Dilutions						
	Undiluted	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$
"Envenomed liver" (0·5 g./c.c.)	2*	2*	2*	2	2	2½	8	—
"Lysocithin" (equivalent to 32 mg. lecithin/c.c.)	½	½	½	½	½	1	2	—

* It was difficult to decide the exact moment of complete haemolysis owing to the opacity of these strong dilutions. Haemolysis probably took place more quickly than is indicated by these times.

When the haemolytic and muscle stimulant activities of "envenomed liver" and of "lysocithin" were compared there was no quantitative agreement. Envenomed liver extracts were proportionately more active upon the gut than by tests of haemolysis. The extract used in the experiments of Table IV in an amount equivalent to 1 mg. of liver had about the same muscle stimulant action as the "lysocithin" in an amount equivalent to 0·3 mg. of lecithin, but compared by haemolysis corresponded with "lysocithin" equivalent to 0·03 mg. of lecithin. This discrepancy may be accounted for by the antihaemolytic action of an alcohol soluble substance or substances present in liver, since normal liver extract exerts a protective effect (Table IV). This, however, does not wholly account for the weaker haemolytic action of "envenomed liver". In one experiment, for instance, in which 1 mg. of "envenomed liver" had approximately the same muscle stimulating action as "lysocithin" equivalent to 200μg. of lecithin, its haemolytic action corresponded to one-quarter of this amount of "lysocithin", even though the "lysocithin" had been tested on the red cells after admixture with extract of normal monkey's liver in amounts equal to those used for testing the haemolytic action of "envenomed liver".

Table IV. Haemolysis times in minutes of washed sheep's red blood corpuscles

		Dilutions					
	Undiluted	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
"Lysocithin"	1	1	$1\frac{1}{2}$	3	10	87	—
"Lysocithin" heated to boiling	1	$1\frac{1}{2}$	$2\frac{1}{2}$	3	11	90	—
"Lysocithin" with normal liver (1 g./c.c.)	—	—	—	—	—	—	—
"Lysocithin" with normal liver (0·1 g./c.c.)	1	2	$4\frac{1}{2}$	15	51	90	—
"Envenomed liver" (1 g./c.c.)	3*	3*	3*	3	3	3	10
"Envenomed liver" boiled (1 g./c.c.)	3*	3*	3*	3	3	$4\frac{1}{2}$	12

* It was difficult to decide the exact moment of complete haemolysis owing to the opacity of these strong dilutions. Haemolysis probably took place more quickly than is indicated by these times.

I. *The perfused liver of the dog. Liberation of histamine*

The injection into the perfused dog's liver of "lysocithin" equivalent to 40–100 mg. of lecithin or of 5–10 g. of "envenomed liver" causes the release of liver pigments, of protein and of histamine and the appearance of these substances in the venous perfusate. The results resemble those obtained by repeated injections of small doses of cobra venom. A first injection of "lysocithin" or of "envenomed liver" was not always followed by the appearance of histamine in the perfusate. In some experiments it produced a histamine output of 40–50μg. and corresponding to more than 1% of the histamine available in the perfused piece of liver;

usually the amounts released were smaller. With successive injections the histamine output increased and after later injections there was an immediate output followed by a gradual and long continued secondary output.

In the experiment of Fig. 29 about 80 g. of liver containing 32μg. of histamine per g. were perfused and eight injections of "lysocithin" were made. The histamine output, which was only trivial after the first injection, increased and became more prolonged after each successive injection. The total amounts assayed were 0·75, 4·3, 13·5, 30·8, 31·7, 42·5 and 75·2μg. After the fourth injection the perfusate, 1 hr. after the injection, still contained histamine and after later injections there was a gradual secondary rise in histamine output. The output of protein increased after the second and third injections and then diminished despite the increase in the output of histamine. The samples collected during the first 10 min. after each injection contained liver pigments. The perfusate collected in the first 3–4 min. after each injection caused slight after-contraction on the guinea-pig's gut, probably from the presence of part of the injected "lysocithin", most of which appeared to be destroyed or absorbed during passage through the liver. The later samples of perfusate caused no after-contraction.

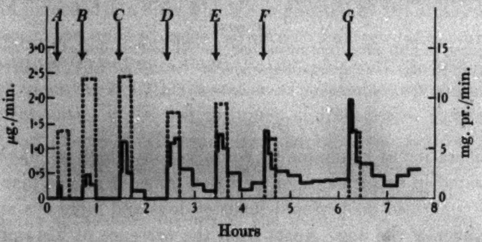

Fig. 29. Output of histamine (continuous line) and of protein (dotted line) from perfused dog's liver. At the arrows, injections of "lysocithin". In this and the following figure the protein output was determined only for the first 15 min. after each injection. Ordinates and abscissae as in Fig. 3. (Details in text.)

"Lysocithin" and "envenomed liver", in doses having the same hæmolytic power, had approximately the same potency for the liberation of histamine (Fig. 30). A piece of dog's liver weighing 80 g. and containing 63μg. of histamine per g. was perfused. At A, C and E "lysocithin" equivalent to 45 mg. of lecithin, and at B, D and F 8·5 g. of "envenomed liver" were injected. The doses of "lysocithin" and "envenomed liver" corresponded in haemolytic power and, as the figure shows, their power of liberating histamine was of the same order. The amounts of histamine in the perfusate after the successive injections were 0·86, 1·6, 11·1, 42·2, 76·7 and 200·5μg.

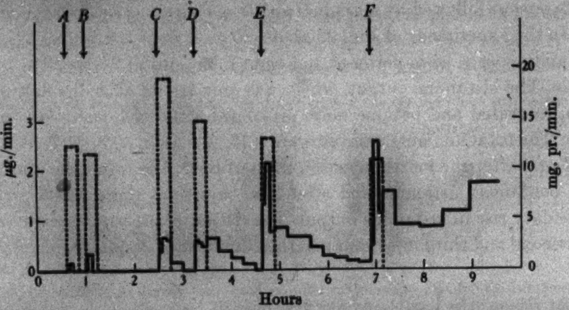

Fig. 30. Output of histamine (continuous line) and of protein (dotted line) from perfused dog's liver. At the arrows, alternate injections of "lysocithin" (A, C, E) and "envenomed liver" (B, D, F). Ordinates and abscissae as in Fig. 3. (Details in text.)

Discussion

The liberation of histamine by cobra venom which we have recently demonstrated in experiments on perfused lungs of guinea-pigs and cats has been confirmed on perfused lungs of dogs and monkeys and on the perfused liver of the dog. Apart from the presence of histamine in the perfusates from envenomed organs, our main interest was concentrated on the appearance of a substance or substances which caused slow delayed contraction of the guinea-pig's gut and unlike histamine must have been formed by the action of the venom in the tissues. The active substance or substances could be extracted with methyl alcohol from the envenomed liver of the monkey and the actions of such extracts were compared with those produced by an alcoholic extract of lecithin treated with cobra venom ("lysocithin"). Both extracts had similar effects on widely different tissues, but there were some quantitative differences. "Envenomed liver" was found to be less active in causing haemolysis and more active in stimulating the gut than was "lysocithin". This difference is only accounted for in part by antihaemolytic substances in liver extracts and makes it uncertain whether the muscle stimulating and haemolytic properties belong to one substance or to two or more. It is even uncertain whether pure lysocithin has any muscle stimulating action. Part at least of the haemolytic activity of both extracts must be attributed to lysocithin and the quantitative agreement between the extracts in haemolytic power, in ability to liberate histamine, and in toxicity for the guinea-pig suggests that all these actions result from the presence of lysocithin. Since the active substances in both extracts appear to be formed by the action of venom on lipins, we shall provisionally regard their total activity as being due to lysocithin-like substances.

In previous communications we have compared the circulatory effects caused by intravenous injection of venom into cats [1937*c*] and into dogs [1937*d*] with those caused by histamine. We found that many symptoms were similar, and since the venom was shown to liberate histamine we concluded that these symptoms could be explained by the action of the liberated histamine. We are now confronted with the fact that venom also causes the formation in the tissues of lysocithin-like substances. Their formation probably represents an intermediate step in the liberation of histamine and precedes it, a view which is supported by the observation that doses of venom too small to cause the output of detectable amounts of histamine cause the formation of small amounts of lysocithin-like substances.

It is often stated that the liberation of histamine represents the first defence mechanism of the cell to injury and that with more intensive damage direct effects of cell injury may dominate the picture. This view has been extended to the cytolytic action of cobra venom. The so-called "direct" effects of the venom may result in part from the action of lysocithin-like substances formed in the tissues. Many effects of cobra venom which cannot be reproduced by histamine may be explained by the action of these substances, which may even contribute directly to the "histamine-like" effects of the venom. We must take into account the fact that these substances act mainly at the site of their formation, whereas when injected they have general effects. This difference may explain some of those actions of "lysocithin" and of "envenomed liver" which are not reproduced by the venom and *vice versa*.

The formation of lysocithin-like substances in the tissues is not confined to cobra venom but plays a part in the symptomatology of poisoning by other snake venoms. Perfusates collected from lungs after the injection of the venom of *Crotalus atrox* and of the Australian copperhead (*Denisonia superba*) contained a substance causing slow contraction of the guinea-pig's gut [Feldberg & Kellaway, 1937*a*], and recently Trethewie has shown that the same is true of the perfusate collected from dog's lung and liver after the injection of the venoms of the Australian black snake (*Pseudechis porphyriacus*) and death adder (*Acanthophis antarcticus*), and that extracts of the envenomed organs are powerfully haemolytic. The formation of lysocithin-like substances in the tissues also occurs with other venoms. The observation of Carpi &

Morgenroth [1906] that the action of bee venom on lecithin leads to the formation of a substance which we can regard as lysocithin, has been repeatedly confirmed, and we have shown [1937 *b*] that perfusate collected from a dog's liver, after the injection of bee venom, contains in large amounts a substance (or substances) which causes slow contraction and transient changes in the reactivity of the guinea-pig's gut.

Other haemolytic poisons may act like snake venoms on the lipins of the tissues. Neuberg & Rosenberg [1907] showed that enzymes acting on lecithin are present in ricin and crotin. Neuberg & Reicher [1907] found that cholera and staphylococcal haemolysin act on lecithin, and we have recently shown that an alcohol soluble substance, which causes slow contraction and after-changes in excitability of the gut, is found in the perfusate of the perfused lungs after the injection of staphylococcal toxin. It is likely that other haemolytic bacterial toxins also form lysocithin-like substances from the tissue lipins.

We must consider the further possibility that this mechanism is not confined to the action of haemolytic poisons but plays a part in less extensive cell injury, such as is brought about by the antigen-antibody reaction of anaphylaxis, by peptone, by ultraviolet light and in short in all those reactions which have been studied on the blood vessels of the human skin by Sir Thomas Lewis and have been attributed to the liberation of *H*-substance. The formation of lysocithin-like substances from tissue lipins in such forms of cell injury would not necessarily be achieved by the direct action of the injurious stimulus but might be brought about by an enzyme set free in the tissue cells. Lysocithin-like substances appear to be easily formed in tissues. Bergenhem & Fåhraeus [1936] have shown that in blood or serum stored at 39° C. a substance appears which they regard as lysocithin formed by lecithinase. These experiments have been confirmed by Bogaert [1937]. Belfanti [1924] showed that pancreatic extracts contain a haemolytic substance which is identical with lysocithin from egg lecithin, and we have found traces of slowly contracting muscle stimulant substance in extracts of normal liver and lungs. This may be an indication that the formation of such substances plays a role in normal tissue activities. There is at present no direct evidence for this more general application of our theory, but there are a few indications in its favour. There are some symptoms common in cell injury produced by non-haemolytic poisons which cannot be explained by liberation of histamine since they are not reproduced by it. We have already mentioned one instance—the reaction of the guinea-pig's uterus poisoned by histamine. The uterus either fails to contract with histamine or actually relaxes, but in this condition it still contracts, not only to cobra venom and to "envenomed liver", but also to peptone and, as Schild [1936] has shown, to the anaphylactic antigen. If we can assume that cell injury is associated with the formation of lysocithin-like substances from tissue lipins many of the inherent difficulties of the histamine theory of cell injury will be removed. The possibility that the muscle stimulating and haemolytic actions of lipin cleavage products are not necessarily associated is favourable to the more general application of this theory of cell injury.

CONCLUSIONS

1. The injection of cobra venom (2–20 mg.) into perfused organs (lung, liver) of dogs and monkeys causes the appearance in the venous perfusate of histamine, of protein and of a substance or substances which cause slow contraction and transient changes in the excitability of the guinea-pig's gut. In the case of the liver, pigments are also set free. No histamine appears in the perfusate of envenomed monkey's liver, since this organ has a very low histamine content. The changes in the venous perfusate from the liver of dogs poisoned by intravenous injections of cobra venom are similar to those observed when the venom is injected into the isolated organ.

2. Histamine, protein and liver pigments are *liberated* from the cells of perfused organs, but the substance (or substances) which causes slow contraction of the gut and subsequent changes in its reactivity is *formed* in the organs by the action of the venom.

3. This substance is present in large amounts in extracts of envenomed organs; it is soluble in absolute methyl alcohol and heat stable. Pharmacological actions of alcoholic extract of envenomed monkey's liver ("envenomed liver") have been compared with those of cobra venom and of extract of lecithin treated with venom ("lysocithin").

4. "Envenomed liver" and "lysocithin" cause slow delayed contraction of the guinea-pig's jejunum and characteristic after-changes in reactivity to histamine and to acetylcholine. The effects of cobra venom are similar, but in this case the muscle is readily desensitized.

5. "Envenomed liver", "lysocithin" and cobra venom contract the rat's jejunum, the normal guinea-pig's uterus and the uterus poisoned by histamine. In the case of cobra venom the preparations are readily desensitized.

6. On the isolated cat's heart "lysocithin" causes changes in coronary circulation and strong reduction in the force of the beat; rapid failure occurs and the heart ceases to beat in diastole or in mid-position. Extracts

of normal monkey's liver, which by themselves have no action, protect from the action of "lysocithin", and if the protection is not complete a gradual failure occurs similar to that caused by "envenomed liver". Cobra venom (2-4 mg.) causes rapid failure and systolic contracture of the heart.

7. Injected into the anterior chamber of the rabbit's eye, "envenomed liver", "lysocithin" and cobra venom cause opacity of the cornea and irregular alterations of its curvature. Extracts of normal monkey's liver are without effect.

8. Injected intravenously into guinea-pigs, "envenomed liver" and "lysocithin" cause symptoms resembling acute anaphylactic shock with the addition of haemorrhagic oedema of the lungs.

9. Washed sheep's red corpuscles are immediately haemolysed by envenomed liver and "lysocithin" but not by cobra venom nor by extract of normal monkey's liver. The latter has a protective action against haemolysis by "lysocithin".

10. "Envenomed liver" and "lysocithin" injected into the perfused dog's liver cause output of protein, histamine and pigments. With repeated injections the output of histamine increases. These effects are closely similar to those produced by repeated injections of small doses of cobra venom.

Our thanks are due to Miss F. E. Williams, who carried out numerous haemolysis tests for us.

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A NEW ARRANGEMENT FOR THE REGISTRATION OF DIAPHRAGM MOVEMENTS

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From the Ciba Research Laboratories

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NUMEROUS curves reproducing the behaviour of the diaphragm have been published during the last few years. The registration of the movements has been made partly with an open thorax and artificial respiration, partly with a closed thorax. The following methods have been used by various authors:

Hess (1936): "For this purpose we used a lever, which has on the lower end a small, flat, hollow dish turned towards the dome of the diaphragm. If suitably placed, the dish follows the movements of the diaphragm. The lever is connected to a Marey's tambour, whose movements are transmitted pneumatically to a tracing capsule which registers the movements."

Verzàr, Szécsényi-Nagy, Haffter, & Wirz, (1937): "For registering the movement of the diaphragm a light spoon made of aluminium foil is sewn flat on to the central tendon of the diaphragm. This was tied over the axis by means of a thread, which traces the movements on a myograph."

Van Voorthuysen & ter Braak (1937): "The diaphragm movements are registered by means of a bent copper lever, which is inserted through an incision in the right abdominal wall between the dome of the diaphragm and the liver. The other end of this lever presses on a tambour, connected by means of a tube to a second tambour placed in front of the slit (in the optical kymograph[1])."

Eichler & Klein (1936): "We opened the abdominal cavity through a longitudinal incision in the linea alba of the animal, introduced a small silver hook into the serosa of the upper muscle fibres of the muscular part of the diaphragm, and connected it to a tracing lever by means of a thread.... The thread was passed through a glass tube, around which the opening in the linea alba is closed up."

Killian & Kuhlmann (1937): "The registration of the movements of the diaphragm is undertaken by means of a special diaphragm spoon by indirect mechanical transmission. The diaphragm spoon is pushed through a slit in the anterior abdominal wall between the top of the liver and the top of the diaphragm. It remained undisturbed for the entire duration of the experiments. The actual registration was undertaken photographically.

In investigating the circulatory action of respiratory stimulants, we were interested in the behaviour of the pressure in the pleural and abdominal cavities in connexion with the movement of the diaphragm. For this purpose, it was necessary for the physiological respiratory mechanism to be disturbed as little as possible and for the bodily cavities to be closed. For these reasons we were not able to use without modification the previously utilized methods. Moreover, it seemed to us to be particularly important to make the movements of the tracer independent of the movements of the musculature. After various attempts, which we carried out with the support of our technical expert, Mr Helmich, we succeeded in devising an arrangement which satisfies the above-mentioned demands. The apparatus is illustrated in Fig. 1. It consists of a lever system which is built into a flat glass vessel. On the front of the lower arm of the lever a light, flat, spoon-shaped piece of copper is attached which is sewn on to the central tendon. If rabbits are chosen as the animals to be experimented on, the use of a very fine arterial needle and silk 00 is necessary for the prevention of pneumothorax. A head-lamp or a reflector facilitates considerably the work, as also does the use of a very long needle-holder and two pairs of large tweezers. The arm of the lever bearing the spoon-shaped piece of copper passes through the lower tube of the glass vessel to the actual registering lever, to which it is connected by means of a joint. The movements are thus transmitted to the lever arm, which is seen in a vertical position in Fig. 1. The lever can

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[1] Not in the original.

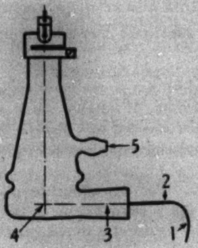

Fig. 1.

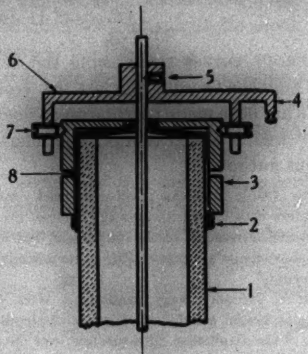

Fig. 2.

Fig. 1. Side view of the diaphragm recorder in the glass vessel. 1, Diaphragm spoon; 2, flat-edged stem; 3, hollow tube; 4, joint; 5, glass inlet for registering the abdominal pressure.

Fig. 2. Section through the top of the tracing apparatus. 1, Glass vessel; 2, thread used for tying on the rubber membrane; 3, metal cap; 4, hook for fastening the thread; 5, screw; 6, metal piece; 7, imaginary axis; 8, rubber membrane.

move quite freely in the triangular glass vessel. Fig. 2 shows the way in which it is mechanically attached, the plane of the diagram being at right angles to that in Fig. 1. The glass vessel is hermetically sealed by means of a thin rubber lamella through which the rod passes. In order to guarantee the greatest possible freedom of movement the rubber is tied on to the rod at the height of the imaginary axis. The rod is fastened to a metal top-piece which forms the external axis. Attached to this metal top-piece is a hook to which the thread leading to the registering apparatus is fastened. The movements can then be recorded on a smoked kymograph or, as in our case, optically. After fixing the lever the glass vessel is sewn into the linea alba and the muscles and skin are carefully adapted to it, if necessary using a rubber cover fixed on the glass vessel. The contrivance for closing the abdominal cavity can be tested to see if it is completely airtight by blowing in air. The registration of the abdominal pressure can similarly be carried out without difficulty, by means of the glass connexion. This appears to us an advantage, since in the case of the system of registration by means of a rubber balloon the direct pressure of the musculature on the latter cannot be excluded. In addition, since it is a closed system its internal pressure is not absolutely uninfluenced by other factors, e.g. temperature. It is true that the fixation of the abdominal wall and of the linea alba to the glass vessel is necessary, but according to our experience this incurs no essential disturbances of the physiological respiratory mechanism.

By means of our apparatus it is possible not only to trace exactly the single movements of the diaphragm but also to observe any alteration in position under physiological conditions. The correct registration of the diaphragm movement and position can be controlled by the abdominal pressure which is recorded at the same time. The mechanical registration of the movement can, moreover, be directly observed in the transparent glass vessel during the experiment. Figs. 3 and 4 are examples of curves obtained. Fig. 3 shows part of a curve obtained with the normal animal which was taken with a rapidly moving kymograph. Without going into details we would refer only to the diaphragm movement, which is represented in proportion to the other registered pressures in the bodily cavities, and the movements of the thorax and of the abdominal wall. The importance of these relations is discussed in more detail elsewhere. Fig. 4 is a curve from which the alteration in tone of the diaphragm by means of histamine can be easily recognized. Further examples of the application of the method are given in a paper by Meier & Müller [1938].

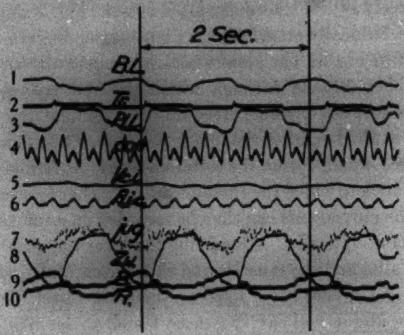

Fig. 3. Registration with rapidly moving kymograph. Normal respiration in the rabbit. 1, Pressure in the upper abdomen; 2, pressure in the trachea (respiration through a valve); 3, pressure in the pleural cavity; 4, blood pressure in the carotis communis; 5, pressure in the vena cava inferior; 6, blood pressure in the arteria iliaca communis; 7, pressure in the vena jugularis externa; 8, diaphragm (inspiration downwards); 9, movement of abdomen; 10, movement of the fifth rib.

Fig. 4. Registration with slowly moving kymograph. Action of 0·1 mg./kg. histamine on the rabbit. 1, Blood pressure in the arteria carotis communis; 2, pressure in the vena jugularis externa; 3, pressure in the vena cava inferior; 4, movement of abdomen; 5, volume respired (measured with a spirometer): the interval between two maxima is an indication of the time in which 3 l. are breathed; 6, diaphragm, inspiration downwards; 7, movement of the fifth rib; 8, pressure in the pleural cavity; 9, pressure in the upper abdomen; 10, pressure in the trachea (respiration through a valve).

SUMMARY

An apparatus is described which makes possible the exact registration of the movements of the diaphragm and its position with closed abdominal and pleural cavities.

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THE FORMATION OF LYSOCITHIN AND OF A MUSCLE-STIMULATING SUBSTANCE BY SNAKE VENOMS

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It has long been known that lecithin treated with venom becomes powerfully haemolytic through the formation of lysocithin. In a previous paper [Feldberg & Kellaway, 1938] the pharmacological actions of alcoholic extracts of envenomed monkey's liver and of a sample of lecithin treated with cobra venom were examined. These extracts, which were shown to be free from venom, were strongly haemolytic, caused slow delayed contraction of the isolated jejunum of the guinea-pig and produced other venom-like effects if applied to other organs. It appeared at first that all these actions might be properties of a single substance, lysocithin, but when a purer preparation of lysocithin was made by one of us (H. F. H.) it lacked the stimulant action on the gut. The effects obtained with extracts of lecithin treated with venom and of envenomed organs had therefore to be attributed to the formation of at least two active substances, and a re-examination of our new lysocithin preparation was necessary. In addition we have studied the action of venom on lymph and on fresh egg yolk.

METHODS

Lysocithin preparation. Lysocithin was prepared by a method based on the work of Levene, Rolf & Simms [1924] and of King & Dolan [1933]. The yolks of twenty-three fresh eggs (approximately 400 c.c.) were washed with distilled water and emulsified with gentle stirring in 400 c.c. 0·11 *M* Na_2HPO_4 adjusted to *p*H 7·4 with HCl. To this emulsion 51 mg. of the venom of the Australian black snake (*Pseudechis porphyriacus*),

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two drops of toluene and 1 g. of thymol were added. The mixture was incubated at 37° C. for 4 hr. A test sample in an amount corresponding to 0·002 c.c. of the mixture haemolysed 0·4 c.c. of 5% washed rabbit red corpuscles at 22° C. After incubation for another hour the mixture was poured into 2 l. of hot 95% C_2H_5OH and filtered immediately. The precipitate was washed with 4 l. 95% C_2H_5OH and the washings added to the filtrate. An excess of saturated $CdCl_2$ aq. was added and the solution cooled overnight in a refrigerator. Next day the precipitated $CdCl_2$ compound was removed by centrifuging and well washed with acetone. It was dissolved in $CHCl_3$ and enough alcoholic NH_3 added to decompose all the compound. The precipitate was removed from the $CHCl_3$ solution and well washed three times with $CHCl_3$. The united solution and washings were concentrated to dryness and the residue dissolved in hot CH_3OH. The CH_3OH solution was cooled in a refrigerator for 3 days, centrifuged while cold and the precipitate well washed with cold CH_3CH. The CH_3OH solution and washings were united and concentrated to dryness *in vacuo*. The residue was dissolved in a little warm chloroform and an excess of ether added. The mixture was immediately cooled and centrifuged, and the precipitate was washed with ether and dried *in vacuo*.

The methods used for the study of the effects of lysocithin were those described in our earlier papers [1937, 1938].

RESULTS

Effect of cobra venom on lymph in vitro

When lymph is acted upon by cobra venom it acquires muscle-stimulating and haemolytic properties.

Lymph was collected from the thoracic duct of a heparinized dog. To 2 c.c. of lymph 100 μg. of cobra venom were added and the mixture kept at room temperature for 1½ hr. Small samples were removed from time to time and were tested on the guinea-pig's jejunum. Such an assay is illustrated in Fig. 1. The gut had previously been treated with three doses each of 20 μg. of cobra venom. When the third dose of venom was added to the bath no contraction occurred, the muscle having become desensitized to doses of venom of this order. At A, 0·2 c.c. of lymph, to which no venom had been added, was tested, it caused slight relaxation. B and C show the effect of the same amount of lymph after venom had been allowed to act on it for 1 and 25 min. respectively. The contraction at B started after a latent period of 25 sec., that at C after a latent period of 14 sec. The contractions could not have resulted from the 10 μg. of venom present in 0·2 c.c. of lymph added to the bath, since the gut was insensitive to twice this dose. The stimulating action of the lymph continued to increase during a further period. The tests at D, E and F were made with 0·02 c.c. of lymph containing 1 μg. of venom only. D was tested 35 min., E 45 min. and F 80 min. after the addition of the venom to the lymph. The increase in stimulating activity is indicated not only by an increase in contraction but also by shortening of the latent period, which was 27 sec. at D, 23 sec. at E, and 17 sec. at F. The stimulating action of the lymph was often followed by characteristic changes in the excitability of the muscle to histamine. These changes were the same as those described in our previous paper [1938] following the addition to the bath of an extract of lecithin treated with cobra venom or of egg yolk treated with venom (*vide infra*).

Fig. 1. Responses of the isolated guinea-pig's jejunum in 5 c.c. bath to lymph before (at A) and after treatment with cobra venom (at B to F). At H, 0·04 μg. histamine. Time in minutes. (Details in text.)

When lymph on which cobra venom had been allowed to act for 2 hr. was extracted twice with absolute methyl alcohol the extract retained its muscle-stimulating property and caused immediate haemolysis of washed human and sheeps' red blood corpuscles. Similarly prepared extract of normal lymph had no immediate haemolytic property.

In 1936 one of us (K.) and Le Messurier had found that dog's lymph, to which small amounts of copperhead venom had been added, caused contraction of the isolated guinea-pig's uterus though the muscle had been desensitized to the venom. This effect and that which we have just described can probably be attributed to the same substance formed by the action of venom upon lymph.

Effect of cobra venom on egg yolk in vitro

When egg yolk is acted upon by cobra venom it acquires muscle stimulating and haemolytic activities. These can no longer be regarded as properties of a single substance, lysocithin, since they can be segregated in different fractions. The haemolytic property must be attributed to lysocithin. The principle responsible for the muscle stimulating effect is also formed by the action of the venom, but we have hitherto not been able to identify it nor to determine the constituent (or constituents) from which it is derived.

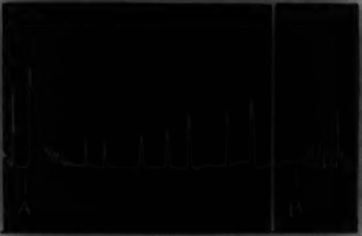

Fig. 2. Responses of the isolated guinea-pig's jejunum to egg yolk treated with cobra venom (at A and B). The rapid responses are to 0·04 μg. histamine. Time in minutes. (Details in text.)

Egg yolk (0·1–0·2 c.c.) has only a feeble and irregular stimulating action on the guinea-pig's gut, but becomes strongly active when venom has been allowed to act upon it. The amounts of venom necessary to produce the effect are small. We found that the addition of 2 μg. of cobra venom to 1 c.c. of egg yolk was sufficient, although the effect became more pronounced when larger amounts of venom were used. Fig. 2 illustrates the effect on the gut of 0·1 c.c. of egg yolk tested 1 hr. after 0·25 μg. of venom had been added to it. The gut had been desensitized to 20 μg. of cobra venom. The contraction at A started after a latent period of 7 sec., and was followed by a period of decreased excitability of the muscle. In some instances this condition was preceded by a short period of increased excitability. If still smaller amounts of venom were added to the yolk, for example 0·5 μg. of venom to 1 c.c., 0·1 c.c. of the mixture caused an increased rhythm for a period of 1–3 min. (Fig. 2 B). In this instance the venom had been allowed to act for 30 min. on the yolk.

For further examination egg yolks treated with cobra venom were extracted with alcohol. For this purpose the yolks of two eggs were incubated for 3 hr. with 12·6 mg. of cobra venom in 30 c.c. of 3% Na_2HPO_4 (*p*H 7·4). The gut-stimulating substance was found to be soluble in absolute ethyl alcohol, and the extracts were powerfully haemolytic on washed sheep red corpuscles. When the alcoholic solution was kept in the refrigerator for 48 hr. a precipitate formed and was removed but the solution still retained most of its gut stimulating and haemolytic properties.

Ether solubility. The alcoholic solution was taken to dryness *in vacuo* and extracted with ether. In the ethereal solution a precipitate formed at once and further precipitation occurred during 48 hr. in the refrigerator. The ether-soluble and insoluble fractions were made up to the same volume and tested for haemolysis and for gut stimulating action. The ether-soluble fraction was about 2½ times more active in stimulating the gut but only half as powerful in causing haemolysis as the ether-insoluble fraction. This indicated that the gut stimulating and haemolytic actions were not properties of a single substance.

Acetone solubility. The ether-soluble fraction when taken to dryness was extracted with acetone, and acetone-soluble and insoluble fractions were obtained. The acetone-soluble fraction was devoid of haemolytic power but was much more active in causing slow contraction of the guinea-pig's jejunum than the acetone-insoluble fraction which was strongly haemolytic. Since lysocithin is known to be insoluble in acetone, the haemolytic power of the acetone-insoluble fraction can be ascribed to lysocithin.

Oleic acid and oleates. The gut-stimulating principle is not only formed by the action of venom on egg yolk and lymph and in perfused organs, but also, as shown in the previous communication, by the action of venom on a preparation of lecithin. It is known that better yields of lysocithin are obtained when venom acts on egg yolk than on lecithin, and in accordance with this fact our egg-yolk extracts had much stronger haemolytic activity than extracts of lecithin treated with venom. The gut-stimulating action of egg-yolk extracts was also much stronger. It appeared therefore that the formation of the gut-stimulating substance was closely associated with lysocithin formation, and it seemed possible that oleic acid or oleates formed in this process might be responsible for the stimulant action on the gut.

We tested oleic acid and sodium oleate in concentrations greater than those which could have been present in our alcoholic extracts of egg yolk and of lecithin treated with cobra venom, and found that they had no stimulating action upon the gut. An 0·5% emulsion of oleic acid in Tyrode solution was added in amounts of 0·2–0·4 c.c. to the bath and sodium oleate in amounts up to 10 mg.

Pharmacological actions of the lysocithin preparation

Sheeps' red corpuscles. Our lysocithin was tested for its haemolytic power by Miss F. E. Williams and found to be active in concentrations up to 1 : 6400, causing complete haemolysis of an equal volume of a 5% suspension of washed red cells within 40 sec.

Guinea-pig's jejunum. Our lysocithin preparation had no stimulating action on the gut, but had an inhibitory influence if given simultaneously with histamine or with an extract containing the stimulating substance formed by venom. When the lysocithin was washed out the jejunum exhibited a period of decreased excitability. In the experiment of Fig. 3 at A and B, 0·1 and 0·5 mg. of our preparation were added to the bath and left in contact with the muscle for 30 sec. After 0·1 mg. the first response to 0·1 μg. of histamine was diminished, after 0·5 mg. the first two doses of 0·1 μg. of histamine were almost without effect and the muscle regained its original excitability in the course of 10 min. After large doses spontaneous rhythm often disappears during the phase of depressed excitability.

We have shown in numerous experiments reported in the previous and in the present paper that the slow delayed contraction of the jejunum produced by extracts of envenomed liver and of lecithin treated with cobra venom is followed by periods of increased and of decreased excitability. The decreased excitability may have resulted at least in part from the presence of lysocithin in these extracts. This would explain the irregularity with which the phenomenon has been observed. We have further shown that the contraction of the gut produced by different venoms, known to form lysocithin, is followed by a period of decreased excitability, and the formation of this lipin-split-product in the muscle may be responsible for this effect. Apparently it is not a direct effect of venom, since a dose of venom which, when added to the bath for the first time, gives the effect, has no such action if added for the second or third time to the then desensitized muscle. This is shown in the experiment of Fig. 3, in which 20 μg. of cobra venom were added to the bath at C and D. The second dose of venom failed to cause any significant contraction of the gut, and there was no after-decrease in excitability. This cannot be explained by loss of sensitivity of the jejunum to lysocithin, because when this was tested subsequently at E and F it had a stronger depressant action than before. Desensitization to the phenomenon of decreased excitability may thus be reduced to the more general phenomenon of desensitization against formation of lysocithin.

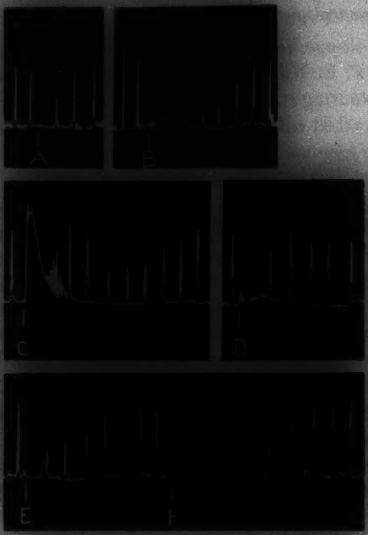

Fig. 3. Responses of the isolated guinea-pig's jejunum to lysocithin (at A, B, E and F) and to cobra venom at C and D. The rapid responses are to 0·1 μg. of histamine. Time in half minutes. (Details in text.)

Fig. 4. Responses of the isolated horn of the uterus of a virgin guinea-pig in a 5 c.c. bath to 2 μg. of histamine (at H) and to 5, 1 and 10 mg. of lysocithin (at A, B and C). Time in half minutes.

Guinea-pig's uterus. The contraction of the virgin uterus which we have described as occurring after extracts of "envenomed liver" and of lecithin treated with cobra venom, must in part at least be attributed to lysocithin. Unlike the intestinal muscle the uterus contracts to our lysocithin preparation in doses from 1 to 2 mg., and the contractions produced by larger doses are followed by a period of decreased excitability. These effects are illustrated in Fig. 4.

Rabbit's eye. The dense and persistent opacity of the cornea, the apparent bulging of the eye from its socket and the associated conjunctivitis which we have described as occurring after the injection into the anterior chamber of extracts of "envenomed liver" and of lecithin treated with cobra venom, must be attributed to the presence of lysocithin in these extracts. The full development of these changes within 12 hr. followed the injection into the anterior chamber of 4 mg. of our preparation of lysocithin. As we have shown, cobra venom produces similar changes which it is reasonable to assume are brought about by the formation of lysocithin.

Intravenous injections in guinea-pigs. The symptoms which we have described in guinea-pigs following the intravenous injection of extracts of envenomed liver and of lecithin treated with cobra venom can be reproduced by the injection of our preparation of lysocithin. After a latent interval of a few seconds there were obstruction to respiration, ineffective respiratory efforts, cyanosis, convulsions, coma and death. In half of the injected animals blood-stained fluid poured out from the nostrils. Post-mortem there was in all cases haemorrhagic oedema of the lungs which did not collapse when the thorax was opened. The intravenous injection of 2 mg. of our preparation produced no symptoms, but 5 mg. caused death in from 1½ to 6 min.

The perfused liver of the dog. Liberation of histamine. The swelling and depigmentation of the liver and the appearance in the perfusate of coagulable protein and of histamine which we have described following the intraportal injection of extracts of envenomed liver and of lecithin treated with cobra venom, must be ascribed to the presence of lysocithin. We were able to produce all these effects with our sample of lysocithin.

After the injection of lysocithin the perfusate became turbid and brownish in tint from liver pigments and sometimes contained a little haemolysed blood in addition. We found that in perfusion experiments lasting from 2 to 3 hr., in the course of which two injections of 100 mg. of our lysocithin preparation were given, the swelling of the organ (as estimated by weighing it before and at the end of the experiment after draining about 10–15 c.c. of fluid) amounted to from 20 to 25%.

Fig. 5 illustrates the output of coagulable protein and of histamine from a piece of liver weighing 60·5 g. and containing about 2·4 mg. histamine. The total output of albumen after the first injection of 100 mg. of our lysocithin (in 2 c.c.) was 0·7 g. The histamine output rose steeply and fell rapidly. At its maximum it was 3·2 µg./min. or 0·13% of the total histamine. A second injection of 100 mg. lysocithin given 1½ hr. later also caused a steep rise which in some experiments reached a higher maximum than after the first injection. But in all cases the total output of histamine after a second was greater than after a first injection and the output per minute remained at a high level for a longer time.

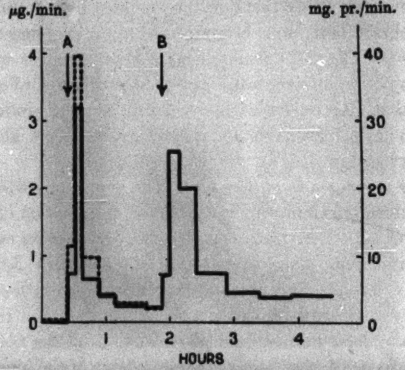

Fig. 5. Output of histamine (continuous line) and of protein (dotted line) from perfused lobe of dog's liver. At A and B, 100 mg. of lysocithin intraportally. Abscissae, time in hours. Ordinates at the left histamine output in µg. per min., at the right protein output in mg. per min.

In our previous communication we have drawn attention to the close resemblance between the histamine output caused by extracts of envenomed liver and of lecithin treated with cobra venom and that caused by repeated small doses (0·3 mg.) of cobra venom. With the larger dose of lysocithin used in the present experiments a striking difference makes its appearance. Whereas the output of histamine after lysocithin rises to a maximum very steeply and subsides fairly rapidly, the maximum output after cobra venom is reached more gradually and may persist at a high level for some hours. In some experiments with bee venom [Feldberg & Kellaway, 1937] a high output lasted from 10 to 15 hr.

There was another difference between the effects of perfusates collected after the intraportal injections of cobra venom and of lysocithin. After cobra venom the perfusates contain large amounts of a slow contracting substance, and the effect can often be elicited with small doses (0·01 c.c.) of these. After lysocithin, though the perfusate was not devoid of such activity, it caused only a moderate effect when doses of 0·2 and 0·4 c.c. were tested. This difference was particularly striking when perfusate after the injection of lysocithin was compared with that obtained when cobra venom was injected into the same liver. Furthermore, when cobra venom was added (50 μg./c.c.) to the perfusate collected after lysocithin and it was tested on a gut which had been desensitized to cobra venom, it was shown to have acquired greatly increased activity and gave strong slow contractions in doses of 0·02–0·04 c.c. Such an experiment on the monkey's liver is described more fully later. We have not ascertained whether the relatively small amounts of slowly contracting substance which appear in the perfusate from the dog's liver after an intraportal injection of lysocithin are liberated or formed.

Monkey's liver. The intraportal injection of our lysocithin preparation caused reticulation of the surface of the liver and depigmentation. There was no perceptible swelling of the organ, and when the liver was weighed at the end of the experiment it had not increased in weight. The venous perfusate became turbid and brownish in colour and contained protein and liver pigments. The perfusate was free from detectable amounts of histamine, which was to be expected, because, as we have shown, the monkey's liver contains less than 1 μg./g. of this substance. No appreciable amount of the principle which causes slow and delayed contraction of the guinea-pig's jejunum could be found in the perfusate. This was not because of any abnormal condition of the liver cells, since perfusate collected after a subsequent injection of a small dose of cobra venom contained the active principle in large amounts. Furthermore, perfusate which, after the injection of lysocithin, was devoid of this activity, rapidly acquired it when treated *in vitro* with a minute amount of venom. These facts are illustrated in Figs. 6 and 7.

Fig. 6 gives the output of protein in mg. per minute after two successive injections each of 100 mg. of our lysocithin (in 2 c.c. of saline) into a lobe weighing 39 g. The output after the first injection reached its peak during the first 15 min. and more quickly still after the second

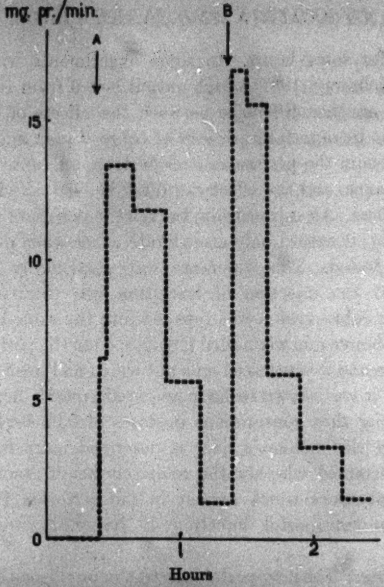

Fig. 6. Output of protein from perfused lobe of monkey's liver. At A and B, 100 mg. of lysocithin intraportally. Abscissae, time in hours, ordinates output of protein in mg. per min.

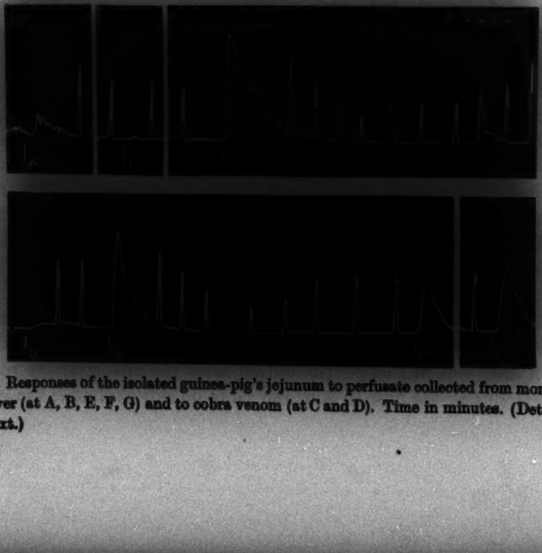

Fig. 7. Responses of the isolated guinea-pig's jejunum to perfusate collected from monkey's liver (at A, B, E, F, G) and to cobra venom (at C and D). Time in minutes. (Details in text.)

injection. The total output after the first injection was 0·45 g., that after the second 0·40 g.

Fig. 7 shows the assay of perfusate from this experiment on the guinea-pig's jejunum. The perfusate collected before the injection of lysocithin was without effect in a dose of 0·4 c.c. At A, 0·4 c.c. of a sample collected between 3 and 15 min. after the first injection of lysocithin was tested. There was a small contraction with a latency of 35 sec. The following samples of perfusate were devoid of activity. At B, 0·4 c.c. of the sample collected in the second quarter of an hour was shown to have no stimulant effect. The gut was now treated with two successive doses of 10 μg. of cobra venom (at C and D). The contraction at C started after a latent period of 8 sec. and was followed by typical changes in excitability. The gut was now desensitized and failed to react at D. At E 0·2 c.c. of the sample of perfusate, which had been tested at B, was retested after incubation with 4 μg. of cobra venom for half an hour. It gave a typical slow contraction of the muscle with a latency of 8 sec. and the characteristic after-changes in excitability. This sample was now active in a dose of 0·04 c.c. (containing 0·8 μg. of venom) at F. The contraction started after a latent period of 15 sec. The newly acquired activity of this sample of perfusate cannot have been due to the cobra venom present in it, but must have resulted from the action of substances formed in it by the venom. The decreased excitability is probably an effect of lysocithin, the delayed contraction and the after increase in excitability to the formation of a muscle stimulant substance. At G is seen the effect of 0·2 c.c. of a sample of perfusate which was collected 5–10 min. after a later injection into the liver of 5 mg. of cobra venom. The contraction was followed by changes in excitability similar to those seen after C and E. It will be observed that the effects at C, E and G are closely similar, and it is reasonable to assume that they are produced in a similar manner by the action of venom on the gut, in the perfusate and in the liver.

Circulatory effects in the cat. The intravenous injection of 20 mg. of our lysocithin preparation after a latent period of 7–10 sec. caused a steep evanescent fall in systemic blood pressure and a concomitant rise of pressure in the pulmonary artery. No desensitization occurred as after the injection of venom, and the blood-pressure changes could be obtained repeatedly. In the experiment of Fig. 8 the effects of three injections of 20 mg. given at intervals of about 5 min. are shown. With larger doses of lysocithin no recovery from the steep fall of systemic pressure occurred; there was a greater rise of pressure in the pulmonary artery and death occurred within a few minutes. Such an experiment is illustrated in

Fig. 9 A. When the systemic blood pressure had fallen, pulsatory oscillations could no longer be seen on the mercury manometer and the record became a thin line. On the other hand, vigorous pulsations were maintained in the pulmonary artery even after the pressure had fallen below its original level. Similar observations were made after the intravenous injection of large doses of snake venom and bee venom. In Fig. 9 is shown for comparison the effects on the systemic pressure and on the pressure in the pulmonary artery of 0·5 mg./kg. of cobra venom (B) and of 100 stings of bee venom (C). The records are indistinguishable from those obtained after lysocithin. The tracings B and C are taken from our previous publications [1937].

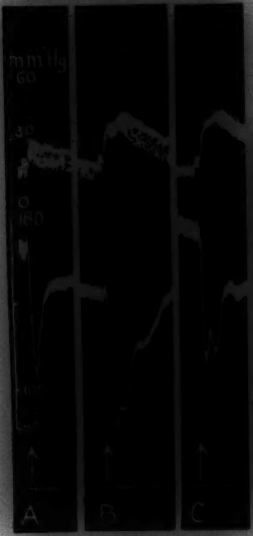

Fig. 8.

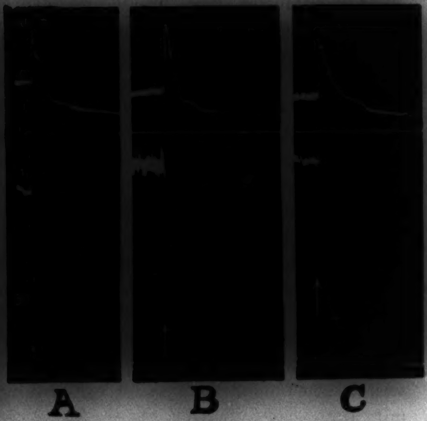

Fig. 9.

Fig. 8. Arterial blood pressure (lower tracing) and pressure in the pulmonary artery (upper tracing) from a cat under chloralose anaesthesia. At A, B and C 20 mg. of lysocithin intravenously. Time in half minutes.

Fig. 9. Arterial blood pressure (lower tracings) and pressure in the pulmonary artery (upper tracings) from three cats. At the arrows 100 mg. of lysocithin (at A), 0·5 mg./kg. cobra venom (at B) and 100 stings of bee venom (at C). Time in half minutes.

The effects of lysocithin resembled those produced by snake venoms and bee venom in another respect. When large doses of these venoms were injected intravenously into cats haemorrhagic oedema of the lungs invariably resulted. Lysocithin produced similar changes. Post-mortem in the experiment of Fig. 9 the lung showed haemorrhagic patches and was tough in consistency. When the survival period was longer, as in the experiment of Fig. 8, there was extensive haemorrhagic oedema of the lung.

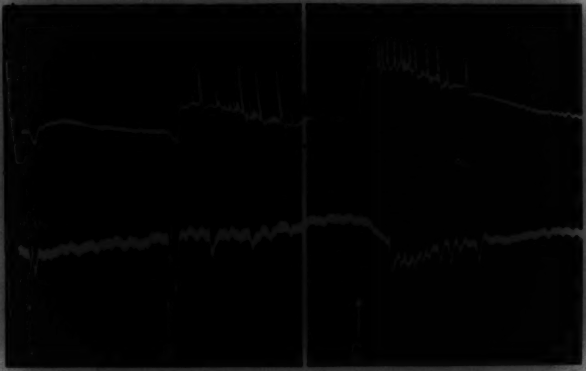

Fig. 10. Arterial blood pressure (lower tracing) and pressure in the portal vein (upper tracing) from a 8 kg. dog under chloralose. At A and B intravenous injections of 50 and 100 mg., at C intraportal injection of 75 mg. of lysocithin. Time in minutes.

Circulatory effects in the dog. The intravenous injection of 50–100 mg. of our lysocithin preparation causes an evanescent steep fall of systemic pressure. There is no desensitization and repeated doses give approximately similar responses. In some instances the fall is interrupted by a short upward fluctuation of pressure (Fig. 10 A and B). The fall in systemic pressure is accompanied by a preliminary slight fall of pressure in the portal vein, which is probably passive, followed by a rather prolonged rise. This results from increased resistance in the liver, since it is stronger when the same or a smaller dose of lysocithin is injected into the portal vein (at C). In this case the rise in pressure in the portal vein is followed by a gradual fall of systemic pressure, which recovers as the portal pressure returns to normal. The fall in systemic pressure in this instance appears to result from the hepatic effects of the lysocithin. The experiment of Fig. 10 shows another effect of lysocithin, which is often observed when snake venoms are injected. The artificial ventilation is overcome by deep spasmodic inspirations, the effects of which are seen in the record of the arterial and particularly of the portal pressure (B and C).

In heparinized dogs we have collected the lymph from the thoracic duct and have injected 100 and 200 mg. of our preparation of lysocithin intraportally and intravenously. There was a transient increase in the lymph flow and a few minutes later the lymph was coloured with pigment from haemolysed blood and contained a few intact red blood cells.

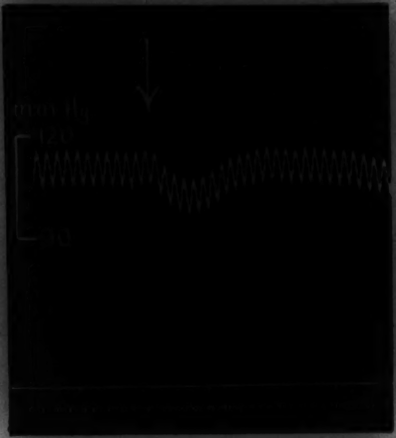

Fig. 11. Arterial blood pressure (upper tracing) time in half minutes (middle tracing) and outflow from the right femoral vein (lower tracing). At the arrow 5 mg. lysocithin injected into the femoral artery.

The evanescent steep fall in systemic pressure produced by the intravenous injection of lysocithin probably results from peripheral vasodilatation. When lysocithin is injected into the femoral artery transient vasodilatation occurs in the limb. After the injection of 1 mg. the dilatation lasted for 20 sec., after 5–10 mg. the outflow from the femoral vein was doubled for 10–20 sec. and had returned to normal within 30 sec. These effects could be obtained repeatedly. Fig. 11 shows a record of the venous outflow after the arterial injection of 5 mg. of lysocithin, each signal corresponding to 3·2 c.c. of outflow.

DISCUSSION

The experiments reported in this and in the preceding paper show that when venom is allowed to act *in vitro* upon lymph, egg yolk or upon a sample of lecithin there is formed in addition to lysocithin a substance which causes slow contraction of the isolated jejunum of the guinea-pig; that these substances are also formed in the tissues by the action of venom on perfused organs and that they may account for many of the symptoms produced by venoms.

The mode of formation of the gut stimulant substance is unknown. Its formation by venom appears to be linked with that of lysocithin, but it is neither oleic acid nor a simple oleate. We do not even know the constituent from which it is derived, which may be another lipin. Its formation in the gut probably accounts for the stimulating action of venom and for the succeeding increase in the excitability of this muscle. Since a similar action on the gut is produced by cell injurious agents which do not form lysocithin it may prove to be an intermediary in these forms of cell injury. In such cases its formation could not be linked with that of lysocithin and it is even possible that it is liberated because a similar substance is present in extracts of normal organs.

The close similarity between the effects of lysocithin and those produced by venoms is easily explained if these actions of venom are caused by the formation of lysocithin, which in some instances acts not only directly but by the liberation of histamine. The formation of lysocithin may continue for some time or it may have a prolonged action in the tissues where it is formed. This would explain the difference in the duration of the effects observed after the injection of venom and of lysocithin into the circulation or into perfused organs. Our experiments with lysocithin have been confined to a few actions only. We have not determined whether it plays any part in the neurotoxic manifestations of venom poisoning. The varying actions of venom on different animals and of different venoms on the same animal may be related to differences in the actions of the phosphatidases of the venoms upon various tissues. Since no desensitization occurs against the action of lysocithin, desensitization against venom must be accounted for by failure to produce further lysocithin in response to a second dose of venom.

CONCLUSIONS

1. Lymph and egg yolk treated with venom acquire a lytic action on red blood cells and cause contraction of the isolated jejunum of the guinea-pig, which has been desensitized to venom. These activities are

the properties of at least two substances. The haemolytic activity must be attributed to lysocithin. The gut stimulant substance has not been identified.

2. Many symptoms produced by venom can be attributed to the effects of these two substances which are formed in the tissues by venom.

3. Lysocithin in addition to its haemolytic action causes decreased excitability of the isolated jejunum and contraction of the uterus of the guinea-pig, opacity of the cornea if injected into the anterior chamber of the rabbit's eye and haemorrhagic oedema of the lung when injected intravenously into the intact guinea-pig. It causes the liberation of protein, pigments and histamine from the perfused liver of the dog, and of protein and pigments from the perfused liver of the monkey. In the cat and dog it causes a steep fall of systemic blood pressure which in the former is associated with a rise of pressure in the pulmonary artery and haemorrhagic oedema of the lung and in the latter with a rise of pressure in the portal vein. Injected into the femoral artery of the dog it causes evanescent vasodilatation. These effects resemble those produced by venoms.

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EFFECTS OF CABBAGE EXTRACTS ON CARBOHYDRATE METABOLISM

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SINCE Collip [1923] described the insulin-like action of injected plant extracts in rabbits and dogs, many attempts have been made to produce substances which when administered by mouth would lower the blood sugar and increase the carbohydrate tolerance of diabetics [e.g. Eisler & Portheim, 1924; Allen, 1927; Leclerc, 1935; Clementi & Torrisi, 1935; Mark & Wagner, 1925; Sippola, 1927; Donard & Labbé, 1933; Laland & Havrevold, 1933]. The presence of a hypoglycaemic principle in plants is supported by a certain amount of clinical evidence. Thus Moscowitz [1937] has reported that diabetics tolerate carbohydrates in vegetables better than corresponding amounts in other foodstuffs. Mori & Seikeichi [1934] show that the hyperglycaemia after the intake of rice is much less when green vegetables are taken at the same time, and so on.

The problem is complicated by the fact that the plant extracts often produce an initial *rise* in blood sugar. Donard & Labbé [1933] held that the presence of maltose in the extract was responsible for this hyperglycaemia, and Labbé [1936] reports that a potent hypoglycaemic extract was obtained from germinated barley after maltose and hordein had been removed. Laland & Havrevold [1933] obtained from onions a crystalline substance which lowered blood sugar in rabbits when mixed with allyl- or diallyl-disulphide, substances which in themselves have no insulin-like action. No extracts have, however, come into general use which can be relied upon when administered by mouth. Insulin modifications and synthetic preparations have proved similarly disappointing when thus exhibited. although certain guanidine derivatives are still extensively advertised as effective.

A preparation which would lower the blood sugar and not exhibit toxic effects when given by mouth would be of therapeutic value. We have, therefore, thought it worth while to reinvestigate the actions of vegetable extracts which have been reported to have such properties. An extract of cabbage has been used in the first instance. Our experiments have so far been limited by the large amount of material which has had to be subjected to extraction in order to yield a few doses of the active material.

METHODS

Several methods for the separation of the hypoglycaemia-producing substance have been evolved. We began our investigations with extracts of spring cabbage and savoy prepared according to the method of Dubin & Corbitt [1923]. The vegetables are minced, extracted with 70% alcohol, and the alcohol is evaporated *in vacuo*. To the remaining aqueous solution, after shaking with ether, four times its own volume of 95% alcohol is added. There separates from this a brown smear which yields a hyperglycaemia-producing extract. To the alcoholic liquor is added three times its own volume of 98% alcohol, and from the precipitate a picrate is prepared. This is decomposed with HCl-alcohol, and further precipitation with ether yields a small amount of a substance which, when administered *per os*, reduces the blood sugar. The extracts were administered to rabbits through a stomach tube. Blood sugar was estimated by the Hagedorn-Jensen method, sugar in the urine by Benedict's method. Glycogen in the liver was estimated by Pflüger's method, slightly modified; all such estimations were made immediately after death.

The rabbits were fed in the mornings on oats, water and greens, but obtained no food for 24 hr. before being used for experiments. The depancreatized dog on which the extract was also tested was given a measured diet—two meals daily of 200 g. minced meat, 30 g. pancreas, 250 c.c. milk, 7·5 g. sugar and a little meat extract. The cabbage preparation was given with this meat extract. At other times the dog was given 7–9 units of insulin hypodermically before each feed.

RESULTS

The hyperglycaemic extract

When doses of this extract equivalent to the yield from 1 kg. cabbage are given by mouth to fasting rabbits, their blood sugar rises rapidly. They begin to breathe heavily, and at the peak of the hyperglycaemia become weak, lie on their sides and often die in 5 or 6 hr. after the feeding. We have found up to 4·6% sugar in their urine, and the livers post-mortem contained only very small amounts of glycogen. Tables I and II give typical results.

TABLE I. Effect of "hyperglycaemic" extracts obtained from 1 kg. cabbage, given by mouth, on the blood sugar of rabbits

	No. 44	No. 46	No. 47	No. 60
Weight (kg.) ...	1·9	1·8	2·2	1·5
Blood sugar (g./100 c.c.)				
Before	0·118	0·120	0·117	0·084
After 1 hr.	0·265	0·236	0·258	0·289
" 2 "	0·401	0·285	0·407	0·460
" 3 "	0·410	0·289	0·444	0·475
" 4 "	0·433	0·360	0·536	0·587
" 5 "	Dead	0·453	Dead	0·543 Dying

TABLE II. Liver glycogen after feeding rabbits with "hyperglycaemic" cabbage extract

	No. 44	No. 47	No. 60
Liver weight (g.)	41	38	32·5
Glycogen total (g.)	0·08	0·16	0·24
Glycogen (g./100 g.)	0·20	0·45	0·74

It appears that, under the action of the extract, the glycogen of the liver is broken down rapidly. When smaller doses (the yield from 500 g. cabbage) are given daily some sustained hyperglycaemia develops. The morning blood sugar may rise to 150–160 mg./100 c.c., as shown in Table III.

TABLE III. Morning blood sugar of rabbits fed with "hyperglycaemic" extract of 500 g. cabbage for five subsequent days

Days	Blood sugar g./100 c.c.
1	0·110
2	0·132
3	0·150
4	0·155
5	0·164

It was found that the extracts contained 21–23 g./100 g. reducing sugar, calculated as dextrose. It seemed unlikely that such a carbohydrate content could produce the blood-sugar changes of Table I. When rabbits were fed with corresponding amounts of glucose, the blood sugar never rose above 212 mg./100 c.c., and had returned to normal in less than 3 hr. No symptoms of hyperglycaemia arose, hence it is concluded that the ingested carbohydrate is not the main cause of the action of the extract. Another possible factor is hydroquinone. Oettel [1936] has found that bilberry leaves contain considerable quantities of hydroquinone which, according to Dietering [1938], causes an increase of blood sugar in the rabbit. Since hydroquinone is extracted from aqueous solutions by ether, and as our extracts had been shaken with ether several times, this also cannot explain our results. Some other hyperglycaemia-producing substance is present in cabbage which can be concentrated by suitable alcoholic extraction.

The hypoglycaemic extract

When this extract, in doses corresponding to the yield of 1 kg. cabbage, is given by mouth to fasting rabbits, the blood sugar falls. This fall varies a good deal in different animals. It usually reaches a turning point in 4 or 5 hr., and the effect may persist for 24 hr. Typical experiments are set out in Table IV. Over forty similar experiments have been made. In none of these have convulsions been observed, nor have other untoward symptoms been detected. A series of experiments has been directed to a comparison of the effects of administration by mouth and by hypodermic injection. No marked difference has been found, nor have we seen any evidence of a reaction to the extract at the point of injection (Table V).

TABLE IV. Effect of "hypoglycaemic" extracts obtained from 1 kg. cabbage, given by mouth, on the blood sugar of fasting rabbits

	No. 11	No. 12	No. 17	No. 18
Weight (kg.) ...	2·3	2·1	2·4	2·1
Blood sugar (g./100 c.c.)				
Before	0·102	0·109	0·100	0·092
After 1 hr.	0·105	0·073	0·090	0·094
„ 2 „	0·102	0·094	0·083	0·082
„ 3 „	0·090	0·075	0·066	0·063
„ 4 „	0·074	0·073	0·045	0·052
„ 5 „	0·062	0·065	0·057	0·071
„ 24 „	0·099	0·071	0·068	0·090

TABLE V. Effect of "hypoglycaemic" extract obtained from 1 kg. cabbage, when given by mouth and by hypodermic injection, on blood sugar in the same rabbit on different days. Weight of rabbit, 2·2 kg.

Blood sugar (g./100 c.c.)	Per os	Hypodermically
Before	0·106	0·112
After 1 hr.	0·103	0·107
„ 2 „	0·096	0·091
„ 3 „	0·085	0·080
„ 4 „	0·071	0·074
„ 5 „	0·071	0·066
„ 24 „	0·077	0·082

For convenience we suggest that the name vegulin may be used for this substance which can be extracted from cabbage and causes hypoglycaemia when given by mouth. The effects of graded doses of vegulin are shown in Fig. 1.

We have made preliminary investigations to determine whether animals develop an early tolerance to the action of vegulin. Rabbits were fed with the extract for 5 days, each dose being the yield of 1 kg. cabbage. During the experimental period they were allowed their ordinary diet, but no food was given in the morning till the sample for blood-sugar estimation had been taken. At the end of this period the response, as set out in Table VI, is not reduced.

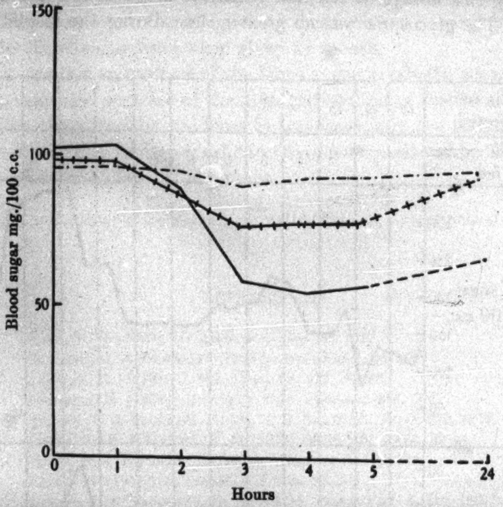

Fig. 1. Effect of different doses of vegulin, given by mouth, on the blood sugar of rabbits. The yield from 0·25 kg. cabbage has little effect; 1 kg. gives a well-marked response, and 3 kg. a profound and prolonged depression of the blood-sugar level. —·— 0·25 kg. ╫╫ 1 kg. —— 3 kg.

TABLE VI. Rabbits fed for 5 days with vegulin. Daily dose: yield from 1 kg. cabbage

	No. 21		No. 22	
Weight (kg.) ...	2·0		1·8	
Blood sugar (g./100 c.c.)	1st day	5th day	1st day	5th day
Before	0·103	0·061	0·107	0·082
After 1 hr.	0·088	0·059	0·096	0·082
„ 2 „	0·051	0·051	0·065	0·073
„ 3 „	0·055	0·049	0·063	0·062

In the depancreatized dog, kept almost sugar-free with insulin, blood sugar varied between 182 and 90 mg./100 c.c. at different hours of the day. When an attempt was made to replace the insulin by the vegulin yield from 3 kg. cabbage, blood sugar rose to 221 mg./100 c.c., but on doubling this dosage, it fell and remained between 154 and 205 mg./100 c.c. The glycosuria was no greater than during the insulin period.

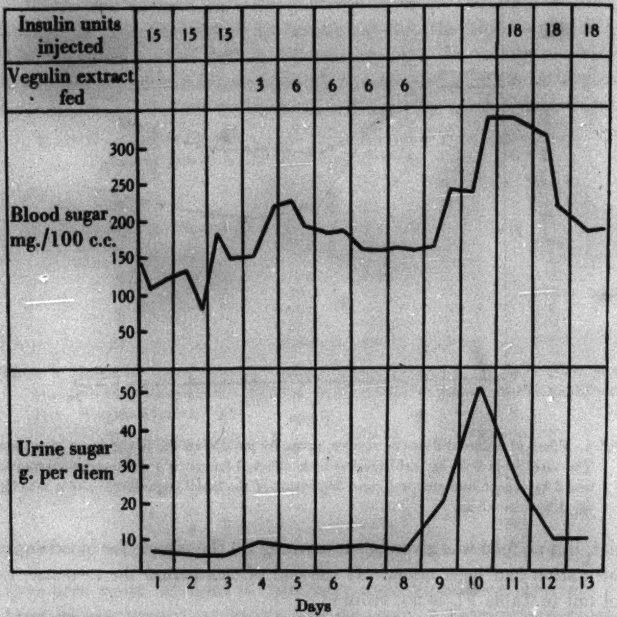

Fig. 2. Action of vegulin on a depancreatized dog. Both blood sugar and glycosuria are well controlled by the vegulin by mouth. When this is discontinued, the diabetic state develops rapidly, but is controlled by resuming insulin on the eleventh day.

On discontinuing the vegulin, blood sugar rose to 320 mg./100 c.c. and glycosuria to 49 g. per diem in 2 days. It appears, then, that the action of insulin in the depancreatized dog can be largely replaced by vegulin (see Fig. 2).

The experiments are being continued with a view to purifying the extract and increasing the yield of active substance. It is also intended to investigate its nature and actions as well as its effect on man and in human diabetes mellitus.

SUMMARY

1. Two fractions of cabbage have been prepared, both of which affect the blood-sugar level when given by mouth.

2. One causes an increase of the blood sugar in rabbits, accompanied by glycosuria and decrease of the glycogen content of the liver.

3. The other fraction produces hypoglycaemia in the normal rabbit and can apparently replace insulin in the depancreatized dog. The name vegulin is proposed for this substance.

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THE TIME OF FUNCTIONAL CLOSURE OF THE FORAMEN OVALE IN THE LAMB

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THAT the foramen ovale is open at birth and that it closes shortly after birth are well-known facts. On the other hand, there appears to be little, if any, reliable information as to the approximate time of such closure. Certain obstetricians have told us that they believe the foramen ovale in the human being ceases to be a functional channel at about 6 weeks from birth.

When accurate knowledge is deficient to such an extent, any additional evidence, however incomplete, is worthy of note, and we therefore present here certain information that we obtained during our studies of the ductus arteriosus in lambs that were delivered by Caesarean section [Barclay, Barcroft, Barron & Franklin, 1938].

Although the ductus arteriosus became functionally closed within 1 min. from delivery, it was clear that the foramen ovale remained patent for a much longer period. The patency was obvious in our experiments, for, when the radio-opaque medium, thorotrast, was injected into either the superior or the inferior caval system, it passed synchronously to the right and left sides of the heart. We noted such passage in every case, and in one instance it was obvious so late as 6 min. 40 sec. from delivery. Unfortunately, it was only possible to determine the direction of flow, and thereby the patency of the foramen ovale, during the initial injection of thorotrast (unless this was a very small one), for the heart chambers were quickly rendered too opaque for further determination of the direction of flow. In addition, our main object was to ascertain the time of closure of the ductus, and for this the interval between delivery of the lamb and injection of the thorotrast had to be reduced to a minimum.

Finally, this same pre-occupation with the ductus caused us to dissect it and neighbouring structures without investigating the patency or otherwise of the foramen ovale, and as luck would have it the season was too far advanced for us to be able to obtain any more ewes in a state of pregnancy. From the ductus experiments, therefore, we were only able to obtain a diagnostic method and evidence of the patency of the foramen ovale up to a few minutes from delivery.

We were, however, able to make one other contribution to the subject, for we made a radiological study of the circulation in a lamb a few hours old, and we afterwards looked at its foramen ovale.

At approximately 8 hr. from its birth, this lamb was prepared for experiment. The wool was cut off over part of the course of the right external jugular vein, the area so exposed was anaesthetized with novocaine solution, and an injection of urethane solution was thereafter made through a cannula tied into the vein. When the subject was adequately under the influence of the narcotic, X-ray cinematographic records were made of the circulation during and following injections of thorotrast. Analysis of these records showed that the foramen ovale was functionally closed at the time of the experiment, for the contrast medium passed out into the pulmonary aorta and its branches a considerable time before any shadow appeared in the systemic aorta.

About 2 hr. later, i.e. about 10 hr. from birth, the lamb was killed with chloroform, the thorax opened, and the right auricle slit up to reveal the interauricular septum. The septum secundum, which was thereby exposed, was divided by a short upward cut, and underneath it the foramen ovale was found completely closed by its valve, a delicate circular sheet of muscle about 1·2 cm. in diameter. When the upper margin of this sheet was gently touched with the tip of a seeker, it became detached for a short distance on either side of the point touched and a circular foramen about 0·6 cm. in diameter was produced as the musculature retracted. Although all pulsation had ceased in the ventricles, the superior vena cava was still contracting every 15–20 sec., and just after each caval contraction the valve of the foramen ovale contracted upwards towards the point from which it had been detached, though the foramen was never completely closed by these post-mortem contractions.

As would be expected from the greater time that had elapsed since birth, the ductus arteriosus in this animal was found to be contracted down more firmly than in any of our previous subjects. Incidentally, as some suggest that the closure of the ductus can be effected by vagal impulses, we had attempted, during the radiological recording, to reopen this channel by intravenous injection of atropine sulphate (0·75 c.c. 1 : 10,000 solution per kg.); in this, however, we met with no success.

We propose, at some future date, to make a cinematographic record of the contractions of the valve of the foramen ovale.

SUMMARY

By means of direct X-ray cinematography combined with injection of radio-opaque solution, it was found that the foramen ovale remained patent, after functional closure of the ductus arteriosus, in lambs delivered by Caesarean section. The latest time at which evidence of such patency was obtained was 6 min. 40 sec. from delivery, but the foramen may have remained patent for a considerable period after this. On the other hand, it was functionally closed in a lamb about 8 hr. old that had been born in the natural way and had breathed normally since birth.

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THE SUPRARENALS AND THE TRANSMISSION OF THE ACTIVITY OF THE SYMPATHETIC NERVES OF THE CAT

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(*Received* 22 *September* 1937)

The progressive failure of retraction of the nictitating membrane of the cat in response to stimulation of the cervical sympathetic nerve trunk after suprarenalectomy was noted in an earlier paper [Secker, 1937]. In that work it became evident that the mechanism of transmission of impulses via sympathetic nerves to the nictitating membrane was dependent on the integrity of the suprarenal bodies, but that the absence of these bodies in no way influenced the response of the membrane to injected adrenaline.

Elliott [1904] found that in the moribund cat, previously suprarenalectomized, certain symptoms were exhibited which were referable to hindrance of those tissues especially innervated by the sympathetics. The tissues so innervated might even fail to respond to electrical stimulation of the sympathetic nerves. These findings led Elliott to suggest that sympathetic axons cannot excite the peripheral tissues except in the presence and perhaps through the agency of adrenaline or its immediate precursor secreted by the sympathetic paraganglia. On the other hand, Elliott [1905] found that the action of the sympathetic nerves on the nictitating membrane of the cat "was neither lessened nor facilitated by previous stimulation with adrenaline". Brodie & Dixon [1904] found that the vasoconstrictor nerves to the limbs or intestine lost their irritability to electrical stimulation in about three hours after death, whereas the vessels reacted to adrenaline six hours after death as fully as they did immediately after slaying the animal. The view suggested by Elliott is not supported by the work of Hoskins & Rowley [1915] who found that "aside from shock, adrenal extirpation produces no immediate effects whatever". They also found that slow infusion of adrenaline, sufficient to raise the concentration in the blood, into dogs, normal or deprived of suprarenals, did not cause facilitation of the responses to sympathetic nerve stimulation. Gley & Quinquand [1919] found that exclusion of the suprarenals from the circulation did not influence the effects of accelerator stimuli on the heart nor did it influence the effects of splanchnic nerve stimulation, or asphyxia on blood pressure.

The purpose of the present investigation was to study the effect of suprarenalectomy on the responses to sympathetic nerve stimulation as exemplified in the retraction of the nictitating membrane, and in the inhibition of tone and movements in the small intestine of the cat.

METHOD

In most of the experiments the cats were anaesthetized with "Dial Liquid Compound, Ciba" (0·08 g. diallylbarbituric acid/kg.) intraperitoneally. In a few cases sodium barbitone (0·4 g./kg.) intraperitoneally was used and in others chloralose (·08 g./kg.) after induction with ether.

Both cervical sympathetic nerves and both vagus nerves were cut and the peripheral ends of the cut preganglionic fibres of the sympathetic nerves enclosed in shielded electrodes for stimulation. The stimulus was obtained by using a variable interrupter in the primary circuit of an induction coil. In the majority of the experiments the current was obtained from a two-volt accumulator and the frequency of stimulation was 30 per sec. Variations in the stimulus in other experiments will be dealt with when those experiments are described.

The suprarenals were excluded from the circulation, in some experiments by ligatures at each pole and a third ligature completely separating the gland from neighbouring structures. In other experiments the glands were completely removed by means of an electric cautery. Usually the glands were removed at the beginning of the experiment, but in some cases the ligatures were placed *in situ* and not tied until after considerable preliminary stimulation of the cervical sympathetic nerves.

The contractions of both nictitating membranes were recorded by the method described in the previous paper [Secker, 1937].

The arterial blood pressure was recorded from either common carotid artery and drugs were given by cannula into the femoral vein.

In studying the inhibition of the small intestine the animals were anaesthetized and, without further interference, lengths of the jejunum or ileum were selected and each length with its mesentery containing the branch of the superior mesenteric artery and nerve plexus was carefully removed. The pedicle of the mesentery containing artery and nerve was enclosed in shielded electrodes and the intestine suspended in Ringer-Locke solution in a Burn and Dale apparatus.

RESULTS

The failure of the response of the nictitating membrane to stimulation of preganglionic sympathetic fibres

The stimulus applied to the nerve trunk was always of sufficient intensity to cause the maximum retraction of which the membrane was capable at the beginning of the experimental period and each stimulus was applied for a period of 30 sec. In the majority of experiments one nerve was stimulated at intervals of 1 min. and the other was occasionally stimulated for purposes of control. In other experiments the nerves were stimulated simultaneously.

When this process of repeated stimulation was carried out in animals possessing their suprarenals, the response of the membrane in most of the experiments was as strong after 2 hr. of repeated stimulation as at the beginning of the period. Repetition of the stimulus in animals deprived of their suprarenals led ultimately to failure of the response. This failure occurred in most male cats fairly rapidly and was usually complete when the stimulus had been repeated 20 times. The number of stimuli necessary to cause this failure varied in different cats and was often quite different for the two cervical sympathetic nerves in the same cat.

This difference in the behaviour of the mechanism in intact (*A*) and suprarenalectomized (*B*) animals is shown in Fig. 1, in each case the nerve has been stimulated 15 times in the interval between tracings 1 and 2. In many of the experiments to be described stimulation of the nerve was discontinued before the response of the membrane had failed completely.

In some experiments the nerves were stimulated 20 times before the suprarenals were removed and the responses were undiminished, but on removal of the glands from the circulation failure of retraction was complete after from 5–10 periods of stimulation. There was no sign of spontaneous recovery when stimulation was discontinued for periods of 30 min.

In female cats the results obtained have not been consistent. In the series of experiments ten female cats were used, and in seven of these the suprarenals were excluded at the beginning. Of these seven cats only one showed complete failure of the response of the membrane after repeated stimulation of the sympathetic trunk, two showed some diminution in the amplititude of the response, and four showed no diminution in the response after repeated stimulation.

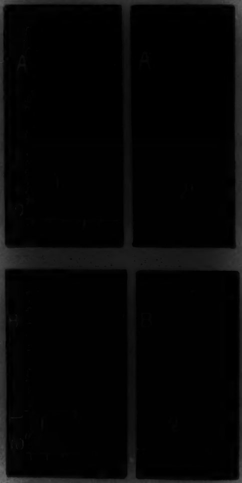

Fig. 1.

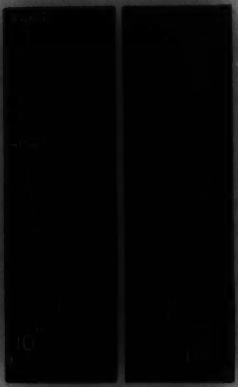

Fig. 2.

Fig. 1. Contraction of the nictitating membrane on repeated stimulation of the cervical sympathetic trunk. *A*. Cat ♀ suprarenals intact (Dial). In the interval between tracings *A*1 and *A*2 (duration 55 min.) the left cervical sympathetic trunk was stimulated fifteen times. *B*. Cat ♂ suprarenalectomized (sodium barbitone). *B*1 was recorded 32 min. after adrenalectomy; *B*2 was recorded 70 min. after adrenalectomy; in the interval between *B*1 and *B*2 the right sympathetic nerve was stimulated fifteen times.

Fig. 2. Cat ♂ suprarenalectomized (Dial). Contraction of the nictitating membrane on repeated stimulation of the cervical sympathetic trunk. Upper record = left membrane, lower record = right membrane. Tracing 1 was recorded 42 min. after, and tracing 2, 64 min. after suprarenalectomy. In the interval between 1 and 2 the left nerve was stimulated eighteen times and the right nerve was stimulated twice as control. In this experiment the blood pressure was recorded from the right common carotid artery.

The remaining three animals of the series were treated differently and require individual description. In one the cervical sympathetic nerve was stimulated repeatedly without any fading of the response, but on removal of the suprarenals the contractions of the membrane diminished progressively to complete failure.

In another experiment the cat was pregnant and the uterus and ovaries were removed along with the suprarenals. In this animal the result obtained was the same as that obtained in the male, i.e. complete failure of retraction to nerve stimulation. The remaining animal, deprived of its suprarenals at the beginning of the experiment, showed no falling off of the response even after 20 periods of stimulation of the nerve; at this stage the ovaries were removed and immediately the response began to diminish until complete failure was attained. This inconstancy of the results obtained with female cats requires elucidation, and further work is in progress.

In all these experiments the condition of the animal was gauged by taking a continuous record of the arterial blood pressure, and in no case was the fall in pressure during the period of the experiment such as to justify the view that the animal was moribund. Fig. 3 records the pressure changes occurring during the period of exhaustion of the response to nerve stimulation.

That the failure of the stimulated neuro-muscular mechanism is due to some local change in the mechanism and is not due to the general condition of the animal is shown in Fig. 2. In this experiment the left nerve has been stimulated to "exhaustion", whereas the right nerve has been used as control, and it is seen that whereas after 20 stimulation periods the mechanism on the left side has failed that on the right side remains unchanged, having been stimulated in all four times.

In the course of this investigation it has been found convenient, for mechanical reasons, to record the arterial blood pressure from one or other of the common carotid arteries rather than from the femoral artery. That the collateral circulation is efficient when one carotid is tied is obvious from the fact that the results described could be obtained from either side of the animal irrespective of which artery was being used to record the blood pressure (Fig. 2).

Stimulation with adrenaline after exhaustion of the nerve mechanism

The retraction of the nictitating membrane to intravenous injection of adrenaline (0·03 mg./kg.) early in the experiment compared with that obtained after the nerve mechanism had failed did not show any appreciable difference in the response (Fig. 12, tracings 1 and 2). It would therefore seem that the "fatigue" or "exhaustion" of the nerve mechanism does not involve the sensitivity of the retractor mechanism of the membrane to adrenaline, thus confirming the previous findings of Secker [1937] and also of Brodie & Dixon [1904].

In the experiments so far recorded the failure of the response followed severe stimulation of a fixed area of the preganglionic fibres of the cervical sympathetic nerve, the nerve trunk being enclosed in shielded, vulcanite electrodes. There are several possible explanations which might be proposed to cover the observed phenomenon, and these possibilities will now be examined.

(1) The failure of the retractor response of the membrane could be due to some change in the local excitability of the nerve trunk:

(*a*) Changes resulting from local damage due to the physical conditions at the electrode. This possibility was explored by substituting a "fluid" electrode [Collison, 1933] for the shielded type, thus obviating the possible results of drying of the nerve fibres. The results obtained when using the fluid electrode were identical with those obtained in the earlier experiments.

(*b*) A condition of diminished excitability of nerve fibres at the point of application of the electrodes and named "stimulation fatigue" by Gotch [1910] might be responsible for the results. This condition of "stimulation fatigue" was investigated by Orias [1932], who found that *continuous* stimulation of preganglionic sympathetic fibres resulted in progressive falling off of the response of the nictitating membrane, but that while shifting the electrodes in either direction caused some recovery to the extent of the response, the contractions did not equal those obtained at the beginning of the experiment and, further, the recovery was short lived. The experiments of Orias were repeated by Dye [1935] who found that the apparent exhaustion of the nerve was due, in part at least, to local "stimulation fatigue", since shifting the electrodes to a fresh portion of the nerve led to an immediate improvement in the response. The recovery observed by Dye, as that observed by Orias, was never such as to give contractions of the initial size.

It is to be noted that in the experiments of Orias and of Dye the animals were in possession of their suprarenal bodies, whereas the animals in the present investigation had been suprarenalectomized.

In my experiments the stimulus (applied for 30 sec. periods) was of such a strength as to give the maximal response of which the membrane was capable at the moment, and this was not always of such an extent as to leave the eye completely uncovered by membrane. In the majority of the experiments to be described in this paper the chosen stimulus strength was maintained and the electrodes were allowed to remain untouched throughout the entire experiment. It was necessary, therefore, to carry out a series of experiments in which the stimulus was varied and the electrodes moved in order to determine how far "stimulation fatigue" was responsible for the failure of the response of the membrane. The results of one such experiment are shown in Fig. 3. In this figure tracing 1 represents the initial response of the nictitating membrane to stimulation of the preganglionic fibres (35 min. after removal of the suprarenals); tracing 2, the response to the 5th period of stimulation with the same strength of stimulus, followed by the response to a period of stimulation of greater intensity (tracing 3). In the interval between tracings 3 and 4 the stimulus was readjusted to the original intensity and applied for three periods and tracing 4 gives the response to the next stimulus applied. During the interval between tracings 4 and 5 the electrodes were moved towards the superior cervical ganglion and tracings 5, 6 and 7 show the responses to three successive periods of stimulation with a stimulus of the original intensity (tracing 5 was recorded 1 hr. after suprarenalectomy). Such results as the above would indicate that "stimulation fatigue" does enter to some extent into the failure of the response of the membrane. The very rapid failure of the response when a fresh site of stimulation is chosen would however suggest that such "stimulation fatigue" is not the main factor in the process.

Fig. 3. Cat ♂ suprarenalectomized (chloralose). Stimulation of the left cervical sympathetic trunk. Upper record contraction of the nictitating membrane; lower record the arterial blood pressure recorded from the right carotid artery. (For description see text.)

(2) The next possible explanation is that the superior cervical ganglion is itself the site of the fatigue. This possibility is easily examined

by applying the electrodes to the postganglionic fibres, where, according to Orias [1932], "stimulation fatigue" does not occur. Fig. 4 illustrates the failure, not carried to excess, of the response of the membrane to repeated stimulation of the postganglionic sympathetic fibres and the recovery of the response under the influence of an extract of the suprarenal cortex (*vide infra*).

Fig. 4.

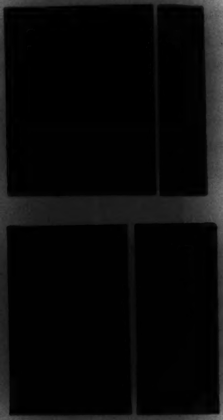

Fig. 5.

Fig. 4. Cat ♂ suprarenalectomized (Dial). Contraction of the nictitating membrane in response to stimulation of post-ganglionic fibres of the left cervical sympathetic nerve at *X*. Tracing 1 was recorded 37 min. after suprarenalectomy andtrac ing 2, 64 min after suprarenalectomy; in the interval between 1 and 2 the nerve fibres were stimulated fifteen times. Tracing 3 recorded 78 min. after suprarenalectomy. In the interval between 2 and 3 "Eschatin" (0·5 c.c./kg.) was given intravenously.

Fig. 5. Cat ♂ suprarenalectomized (Dial). Effect of continuous stimulation of the right cervical sympathetic trunk at a frequency of 12 per sec. on the right nictitating membrane. Tracing *A* was recorded 40 min. after adrenalectomy and occupied 7 min., the interval between *A* 1 and *A* 2 = 3 min. In the interval between tracings *A* and *B* "Eschatin" (0·3 c.c./kg.) was given. Tracing *B* was recorded 65 min. after adrenalectomy and occupied 22 min., the interval *B* 1–*B* 2 = 18 min.

(3) Orias found that continuous stimulation of preganglionic fibres with a frequency of 42 stimuli per sec. led to relaxation of the nictitating membrane due to a process of inhibition described by Wedensky and depending on the refractory period of the ganglion itself. Changing the frequency of stimulation to 3 stimuli per sec. caused a recovery of

the extent of contraction which persisted as long as the exciting stimulus. The same result was obtained with stimuli of frequencies up to 20 per sec.

Since the stimulus normally used in the experiments described in the present paper had a frequency of 30 per sec. and was applied for a period of 30 sec. it was thought advisable to study the effect of continuous stimulation with stimuli of a frequency of less than 20 per sec. in suprarenalectomized animals. Accordingly in three experiments the preganglionic fibres were stimulated, using a fluid electrode, at a frequency of 12 per sec. using 6 V. in the primary circuit.

Tracing *A*, Fig. 5, shows the effect of this procedure, i.e. a rapid failure of the contraction of the membrane. In this experiment the process was allowed to go on to almost complete exhaustion, the interval between tracings *A* 1 and *A* 2 covered 3 min. of continuous stimulation. The stimulus was stopped and the animal treated with extract of the suprarenal cortex (*vide infra*).

Tracing *B* shows the effect of prolonged stimulation after treating the animal with cortical extract. The interval between tracings *B* 1 and *B* 2 represents 18 min. continuous stimulation. The electrode was not moved during the course of the experiment.

The results of these and similar experiments support the view that the neuro-muscular mechanism of the nictitating membrane is adversely affected by removal of the suprarenals. The results seem to justify the belief that some substance liberated by the suprarenal bodies is essential for the continued action of sympathetic nerves. It is possible that with repeated excitation of the nerve, adrenaline which has in some way been stored by the nerve mechanism (nerve fibres or myoneural junction) has been used up.

Effect of injected adrenaline on the response to nerve stimulation

When in a suprarenalectomized animal the nerve was exhausted, or almost so, and adrenaline (0·03 mg./kg.) was administered the membrane responded as usual by retraction and then relaxed. If within a minute of relaxation the nerve was stimulated the membrane again responded but not to its original extent. Further stimulation was generally ineffective. Fig. 6 shows the diminution in the response to nerve stimulation and the transient beneficial effect of injected adrenaline. Tracing 1 gives the initial response of the membrane to the stimulus 52 min. after suprarenalectomy; in the interval (6 min.) between 1 and 2 the nerve was stimulated five times; in the interval between 2 and 3 (4 min.) the stimulus was applied twice and tracing 3 shows the absence of response by the membrane. In the interval between 3 and 4 adrenaline was given and the resultant contraction allowed to pass off. Tracing 4 shows the slight recovery of the response on stimulation of the nerve immediately after the adrenaline contraction was over. The interval between 4 and 5 was 1 min. and tracing 5 shows that the effect of adrenaline on the response to nerve stimulation is very transitory. Tracing 6 will be dealt with later. Such results show that the small amount of adrenaline remaining after the response to injection is in some way capable of reviving the neuro-muscular mechanism, but only to a slight extent and for a short period of time. It is possible that the adrenaline available for "storage" in the time is too small in amount to give any further recovery.

Fig. 6. Cat ♂ suprarenalectomized (Dial). The influence of adrenaline (tracing 4) and of "Eschatin" (tracing 6) on the response of the nictitating membrane to stimulation of the "exhausted" left cervical sympathetic nerve. (For description see text.)

If this view is correct it should be possible by continuous intravenous infusion of adrenaline to maintain or further increase the improved responses to nerve stimulation. With this idea in mind suprarenalectomized cats, in which the neuro-muscular mechanism of the membrane had been partially exhausted by repeated stimulation of the nerve trunk, were given continuous infusions of adrenaline (1 in 200,000 solution) into the femoral vein at the rate of 1 c.c. per min. This infusion produced a sufficient concentration in the blood to maintain the arterial blood pressure at a slightly higher level. Stimulation of the nerve during this period of infusion resulted in a definite improvement in the response of the membrane and the improvement persisted for a short time after termination of the infusion and return of the blood pressure to its pre-adrenaline level.

Fig. 7 records the result of one such experiment. In this experiment the position of the electrodes and the stimulus remained unchanged.

The upper record in each tracing shows the extent of contraction of the membrane in response to stimulation of the nerve trunk. The lower record gives the level of the arterial blood pressure. Tracing 1 records the response at the beginning of the experiment 56 min. after suprarenalectomy; in the intervening 11 min. between tracings 1 and 2 the nerve was stimulated seven times. During the interval between 2 and 3 the adrenaline infusion was started and tracing 3 was taken after the infusion had been going for 4 min. Tracing 5 was recorded 30 sec. after the infusion had been stopped. In the interval between 5 and 6 which represents a period of 6 min. the nerve had four periods of stimulation.

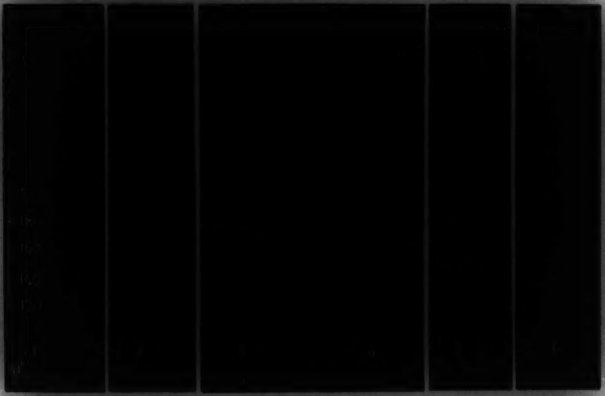

Fig. 7. Cat ♂ suprarenalectomized (Dial). The influence of slow continuous infusion of adrenaline on the response of the nictitating membrane to repeated stimulation of the left cervical sympathetic trunk. Blood pressure recorded in mm. Hg. (For description see text.)

Pursuing the idea of "stored" adrenaline still further use was made of the findings of Bacq [1936], "that certain substances which, *in vitro*, protect adrenaline from oxidation" (e.g. pyrogallol) potentiate and prolong its action *in vivo*, and of Burns & Secker [1936] "that the inactivation of adrenaline is slowed down in the presence of guanidine salts".

In three experiments the nerve mechanism of the membrane was exhausted after suprarenalectomy and the animal was given pyrogallol (10 mg./kg.) and the nerve again stimulated without effect (Fig. 8, tracings 2 and 3). Administration of adrenaline (0.03 mg./kg.) was followed by the typical more prolonged post-pyro. contraction and when this had passed off stimulation of the nerve showed that recovery had

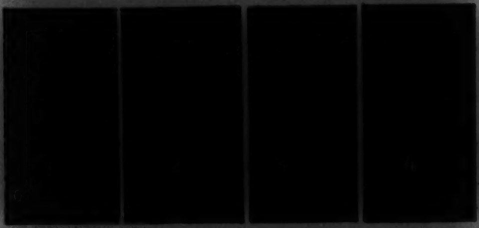

Fig. 8. Cat ♂ suprarenalectomized (sodium barbitone). Contraction of the nictitating membrane on stimulating the left cervical sympathetic trunk. (1) 20 min. after suprarenalectomy; (2) after an interval of 5 min. during which the nerve was stimulated for three periods; (3) 18 min. later, during the interval 20 mg./kg. pyrogallol had been given; (4) the effect of adrenaline. Adrenaline (0·3 mg./kg.) was given 5 min. before the record was taken. The interval between tracings 3 and 4 = 20 min.

Fig. 9. Cat ♂ suprarenalectomized (sodium barbitone). Contraction of the left nictitating membrane on stimulation of the sympathetic nerve; (1) 60 min. after suprarenalectomy; (2) after an interval of 45 min. which included fourteen stimulation periods; (3) 5 min. after guanidine carbonate (50 mg./kg.) had been given; (4) 10 min. after 0·01 mg./kg. adrenaline.

taken place (Fig. 8, tracing 4). The recovery of the mechanism was more marked in these cases than where adrenaline was given without previous treatment with pyrogallol and the recovery persisted for a longer time, usually requiring four or five stimuli to exhaust again, and after this re-exhaustion the action of the nerve could be restored by a further injection of adrenaline. In other experiments a similar method was followed and guanidine carbonate (50 mg./kg.) was given after complete or partial exhaustion of the nerve mechanism, here again without appreciable effect on the response to nerve stimulation. But when adrenaline was given (0·01 mg./kg.) the recovery of the response was marked and persisted for a longer time than after pyrogallol (Fig. 9). However, it was again possible to exhaust the mechanism and to obtain further recovery by repeating the dose of adrenaline.

This recovery of the response to nerve stimulation in the presence of adrenaline is certainly obvious when the hormone is given by continuous infusion, but the extent of recovery is still greater when adrenaline is artificially protected by pyrogallol or guanidine, and it is arguable that no such protection is known to exist in the body and, since the beneficial effect of injected adrenaline is so short lived, that protection obviously does not exist in the animal deprived of its suprarenals.

The cortex of the suprarenal and the activity of sympathetic nerves

Removal of the suprarenals deprives the animal not only of its chief, if not sole, source of adrenaline but also of the products of the cortical portion of the glands. It has been shown that adrenaline administered in sufficient quantity to raise the blood pressure does give some improvement in the response of the membrane to nerve stimulation, but it does not give anything like complete restoration of the neuro-muscular mechanism. It was therefore necessary to study the effect of administration of extracts of the cortex of the suprarenal. The preparation used was "Eschatin" (Messrs Parke, Davis and Co.), which is an extract of the suprarenal cortex prepared by the method of Swingle & Pfiffner [1931]. In this series of experiments the sympathetic nerve was stimulated, after removal of the suprarenals, until the nictitating membrane failed to respond, or in some cases until there was marked diminution in the response, "Eschatin" (0·05–0·5 c.c./kg.) was then given intravenously and the nerve again stimulated. Usually the extract required about 5–10 min. to develop its action, which showed itself in a complete recovery of the mechanism to nerve stimulation (Figs. 4, 5, 6 and 10).

Fig. 10 shows one such result when "Eschatin" (0·1 c.c./kg.) was given in the interval between tracings 2 and 3. It will be noted that in this experiment the extent of contraction is slightly greater after "Eschatin" has been given than it was in the original response shown in tracing 1. The explanation is, I think, that in suprarenalectomized animals there is some loss of efficiency of the neuro-muscular system during the time interval which occurs between removal of the glands and the beginning of actual recording, and therefore even supramaximal stimuli cannot cause complete retraction of the membrane.

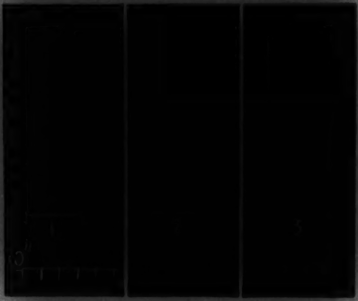

Fig. 10. Cat ♂ suprarenalectomized (Dial). Contraction of the left nictitating membrane on stimulation of the cervical sympathetic nerve. (1) 38 min. after suprarenalectomy; (2) after an interval of 14 min. in which the nerve was stimulated for twelve periods; (3) 16 min. later and 10 min. after 0·1 c.c./kg. "Eschatin" was given.

Fig. 4 shows the recovery of the response to stimulation of post-ganglionic fibres after giving (0·5 c.c./kg.) "Eschatin" during the interval between tracings 2 and 3.

Fig. 5 shows strikingly the effect of the cortical extract in prolonging the contraction of the membrane under continuous stimulation of low frequency. In the interval between tracings *A* and *B* "Eschatin" (0·3 c.c./kg.) was given.

Fig. 6 shows recovery of the response under the influence of cortical extract after an attempt at recovery under the influence of adrenaline. "Eschatin" (0·05 c.c./kg.) was given in the interval between tracings 5 and 6 (70 min. after suprarenalectomy and tracing 6 was recorded 6 min. later). It is necessary to note that "Eschatin" itself does not cause any response on the part of the nictitating membrane unless given in massive doses.

The recovery under the influence of the cortical extract is without the aid of injected adrenaline and persists for a considerable time which varies with the dose given. If a sufficient quantity has been given it may require as many periods of stimulation to exhaust again as were necessary for the first exhaustion.

Fig. 11 is from the same experiment as Fig. 10 and commences after a rest period of 30 min., 21 stimulation periods were required to exhaust the mechanism to the stage shown in tracing 3 (Fig. 11) and recovery was again obtained by giving 0·1 c.c./kg. of the extract in the interval between tracings 3 and 4.

Fig. 11. From the same experiment as Fig. 10. (1) Contraction of the membrane on stimulation of the nerve 30 min. later than tracing 3 (Fig. 10); (2) after twelve periods of stimulation; (3) after a further nine periods of stimulation; (4) after a further injection of "Eschatin" (0·1 c.c./kg.).

Cortical extract and the response of the membrane to adrenaline

The response of the nictitating membrane to injected adrenaline in adrenalectomized animals is not increased by the administration of cortical extract. Fig. 12 shows the response of the membrane to adrenaline (0·03 mg./kg.) (1) at the beginning of the experiment 43 min. after adrenalectomy; (2) 24 min. later after the exhaustion of the nerve; (3) 16 min. later and 6 min. after giving "Eschatin" (0·05 c.c./kg.). The mechanism had recovered its response to nerve stimulation before tracing 3 was recorded.

Inhibition of the isolated loop of intestine

The results obtained with the nictitating membrane in the adrenalectomized animal ought to be capable of confirmation by isolating some structure innervated by a sympathetic nerve. The isolated loop of small intestine is a convenient test object for this purpose. Stimulation of the mesenteric nerves to the isolated intestine for 30 sec. resulted in diminution of tone in the muscle and inhibition of the rhythmic movements.

Repetition of the stimulus resulted in gradual failure of the inhibition until stimulation of the nerve was without effect. Increasing the strength of the stimulus did not cause reappearance of inhibition. Addition of 0·5 c.c. "Eschatin" to the bath containing the intestine in 250 c.c. Ringer's solution resulted in recovery of the inhibitory effects of nerve stimulation. When the bath was emptied and the Ringer's solution renewed the inhibitory mechanism could again be exhausted and further recovery obtained by the addition of a fresh supply of the cortical extract.

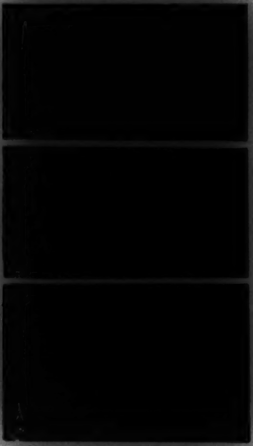

Fig. 12.

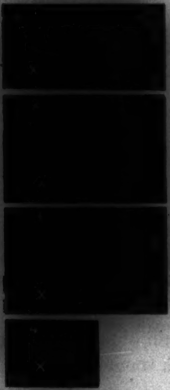

Fig. 13.

Fig. 12. Cat ♂ suprarenalectomized (Dial). Contraction of the right nictitating membrane on injection of adrenaline (0·03 mg./kg.) at *A*. (For description see text.)

Fig. 13. Inhibition of the isolated loop of small intestine on stimulation of the mesenteric nerves at *X*. (For description see text.)

The results of one experiment are shown in Fig. 13. Tracing 1 is the effect of stimulation of the nerve at the beginning of the experiment, and in the interval between tracings 1 and 2 (duration 15 min.) the nerve was stimulated eight times. Tracing 2 shows the marked falling off of the inhibitory response to nerve stimulation. It will be noticed that in this record the rhythmic movements have increased in amplitude, this being a common but not invariable result of exhaustion of the nerve. Tracing 3 shows the recovery of the inhibitory response under the influence of 0·5 c.c. "Eschatin" which was added to the bath in the interval (duration 5 min.) between tracings 2 and 3. In the interval between tracings 3 and 4 (duration 10 min.) the fluid in the bath was renewed and the nerve stimulated three times. Tracing 4 shows the complete exhaustion of the inhibitory response.

DISCUSSION

That the progressive failure of the response to excitation of pre-, or postganglionic sympathetic nerve fibres is not due to any local condition of diminished excitability seems evident from the results recorded above.

In the first place, while there is evidence that "stimulation fatigue" does enter into the process, the recovery observed on changing the site of stimulation is so transitory that it does not seem possible that this condition can be responsible for the observed facts. Further, in certain experiments, not recorded above, the stimulation has been carried to exhaustion and the electrodes moved successively in either direction and no response has been obtained, yet on administering cortical extract the response has reappeared.

Secondly, in the series of experiments represented by that from which Fig. 5 is taken, the stimulus was of such a frequency that this process of "stimulation fatigue" is said to be avoided. In these experiments when cortical extract was given the stimulus could be applied for considerable periods before any sign of failure appeared.

Finally, the fact that "Eschatin" causes recovery no matter what the type of stimulus used to produce the failure would suggest that this failure is chemical in origin.

The results reported would seem to give definite evidence that sympathetic nerves in the cat, whether of the excitor type as exemplified in the supply to the nictitating membrane, or of the inhibitor type as supplied to the muscle of the small intestine, require the influence of the suprarenals for their continued function. Further, that under normal conditions the "nerve-muscle" system possesses a store of some substance which is apparently maintained under the influence of the suprarenals. After removal of the suprarenals this store can be exhausted by repeated excitation of the system through its nerve fibres, and the glands being absent the deficit is not made good. It is to be remembered that in these experiments the rapid exhaustion of the sympathetic mechanism is the result of severe excitation, and that while in these experiments there was no sign of unaided recovery in the period of the experiment there is nothing to show that some adaptation might not have occurred in a longer period such as we have in the less acute two-stage removal of the suprarenals.

The inconstancy of the results obtained with female cats seems to point to some functional relationship between the suprarenals and the ovaries. It is of interest to note that Rogoff & Stewart [1928] demonstrated that dogs adrenalectomized during or shortly after oestrus survive for a much longer period than animals in dioestrus. The explanation of this fact given by Grollman [1936] is that oestrus stimulates the suprarenals to produce more hormone for storage in the body than is normally present.

When we remember the complexity of the hormonic activities of the ovary and the variation of these activities during the different phases of the sex cycle it is not justifiable, without a much deeper investigation, to make any speculation as to this relationship but simply to note that the intact female *may* give results similar to the male and that the spayed female *does* give these results.

Since the retractor muscle of the nictitating membrane is still capable of giving an undiminished response to injected adrenaline when the "nerve-muscle" system is exhausted it is obvious that the failure of the response to nerve stimulation is not due to fatigue of the muscle, but is due to some defect in the nerve itself or in the myoneural junction. This defect is apparently a deficit of the substance which normally transmits excitement from the nerve fibres to the muscle or the precursor of this substance. Since immediately after suprarenalectomy there is a sufficient quantity of the transmitter to give many responses on the part of the muscle, it is logical to assume that as free adrenaline in this quantity would cause contraction of the membrane of its own accord the missing substance is not adrenaline in the free state.

As the absence of the transmitter does not interfere with the ability of injected adrenaline to excite the muscle, we must, if we accept adrenaline as the sole transmitter of sympathetic activity, conclude that the fault is one of the following pair: (1) that the missing substance is adrenaline which is normally taken up in minute quantities and stored, to be liberated under the influence of nerve impulses, or (2) that it is the precursor of adrenaline, normally stored, but activated and liberated by the action of nerve impulses.

If we do not accept this view of adrenaline as the sole agent in sympathetic activity there remains the possibility that structures innervated by the sympathetics have a dual excitability, i.e. to adrenaline and to some transmitting agent, not adrenaline, liberated by nerve impulses, and that the missing substance is this transmitter or its precursor. If we examine these possibilities we find in the experiments in which the previously exhausted nerve was stimulated after adrenaline there was a slight transitory recovery, suggesting that some part of the system, presumably the myoneural junction, was capable of taking up and holding adrenaline which could be liberated by nerve impulses to cause contraction of the muscle. The quantity so held was either too small, or the conditions under which it was held lacked stability, since its efficacy quickly diminished. The use of substances like pyrogallol and guanidine which are presumed to delay the inactivation of adrenaline certainly do increase the extent and duration of the recovery following the injection of adrenaline. This result could follow from the protection of adrenaline allowing a longer period for the taking up of minute amounts and the rendering of the adrenaline so taken up more stable.

At the moment we are not aware of any substance normally present in the body which is capable of giving this protection to adrenaline.

A much more likely explanation of the phenomenon is that immediately after the response to injected adrenaline there remains in the blood, or at the myoneural junction, subthreshold quantities of the hormone and that on stimulation of the incompletely exhausted nerve (using the term nerve to include myoneural junction) subthreshold amounts of the transmitter are liberated and that as the result of summation the muscle is excited. In the presence of pyrogallol or guanidine these subthreshold quantities of adrenaline will be present for a greater length of time.

The remaining possibilities are that the depleted substance is the precursor of adrenaline or some specific transmitting agent or its precursor. There is nothing in the results of these experiments which helps to differentiate between these possibilities. Examination of the experiments in which an extract of suprarenal cortex was given shows complete recovery of the "nerve-muscle" system, and this recovery was maintained for varying lengths of time according to the quantity of extract given and the amount of subsequent stimulation. The extract itself does not contain a sympathomimetic substance since it does not cause any appreciable effect on blood pressure, nor does it cause contraction of the nictitating membrane, and therefore the recovery cannot in this case be due to summation. It seems that the extract must contain either (*a*) the element of which the system is depleted on repeated excitation following the removal of the suprarenals, or (*b*) some substance concerned in the reformation of this element. Obviously the material contained in the extract is neither adrenaline nor a specific transmitter of sympathetic activity, since it does not have a sympathomimetic action and it is more likely to be concerned in the formation of one or other of these substances. It is not a substance which protects adrenaline since the recovery of the mechanism occurs without the aid of adrenaline, and further it does not in any way increase or prolong the response of the nictitating membrane to injected adrenaline. This recovery of the sympathetic nervous system under the influence of cortical extract is in conformity with the great mass of work which tends to demonstrate that it is the cortex and not the medulla of the suprarenal body which is indispensable for life. The fact that cortical extracts free from adrenaline can maintain suprarenalectomized animals in apparently normal physiological condition is convincing proof of the necessity for the hormone or hormones produced by this part of the gland. The experiments on the isolated loop of small intestine confirm the main facts elucidated in the experiments on the nictitating membrane in that they show again the fact of a store of the precursor of the transmitting agent which can be depleted on repeated stimulation of the mesenteric nerve and is replaced under the influence of cortical extract. These experiments are of interest in that they demonstrate a mechanism for inhibition by sympathetic nerves similar to that pertaining to excitation.

SUMMARY

1. Repeated stimulation of the cervical sympathetic nerve in suprarenalectomized cats leads to failure of the response of retraction by the nictitating membrane.

2. Adrenaline gives a slight and temporary recovery of the response to nerve stimulation.

Pyrogallol and guanidine which are presumed to "protect" adrenaline increase and prolong this recovery under adrenaline.

3. Extracts of the cortex of the suprarenal restore the function of the exhausted nerve. This restoration can be overcome by further prolonged excitation and the nerve again restored by more extract.

4. The inhibitory action of the mesenteric nerve on the isolated intestine is also exhausted by repeated stimulation and can be revived by cortical extract.

I wish to express my thanks to Prof. D. Burns for his interest during the progress of this work. I have also to acknowledge, with thanks, that the expenses involved were partially defrayed by a grant from the Medical Research Council. My thanks are also due to Messrs Parke, Davis and Co. who supplied the first samples of "Eschatin".

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THE BLOOD-PRESSURE RAISING SECRETION OF THE ISCHAEMIC KIDNEY

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The co-existence of arterial hypertension and renal lesions has been observed for a long time. This fact has induced many investigators to attempt the production of arterial hypertension by experimental modification of the kidney. The methods used have been numerous and varied, e.g. surgical removal of part of the kidney, ligature of the renal artery or some of its branches, reduction of the calibre of the renal vein, ligature of the ureter, multiple emboli of the kidney, irradiation of the kidney, toxic nephritis, renal compression, etc. References to the principal experiments of this nature can be found in the articles by Braun-Menéndez [1932], Goldblatt [1937] and Fasciolo [1938*b*].

In 1927, with Biasotti, experiments were done on partial removal of the kidney, and in 1933 with Braun-Menéndez on partial occlusion of the renal vein in the dog, but hypertension so obtained was generally inconstant and transient, therefore the experiments were discontinued. Eventually satisfactory and constant results were obtained using the technique of Goldblatt, Lynch, Hanzal & Sumerville [1934], compressing the renal artery and reducing its calibre by means of an adjustable forceps, which caused renal ischaemia and permanent hypertension with 50–100 mm. Hg rise above the initial blood pressure. The arterial tension generally rose from an initial level of 130–140 to 180–250 mm. Hg; 185 animals thus treated were studied.

Compression of the splenic or femoral arteries [Goldblatt *et al.* 1934] or the mesenteric or coeliac arteries [Houssay & Fasciolo, 1937*b*, 1938] did not produce hypertension; that of the coeliac trunk produced a gradual hypotension in two cases.

The arterial tension was measured directly by arterial puncture or with Van Leersum's arterial loop or the indirect technique of Biasotti. In the end the first was the principal method used.

ARTERIAL HYPERTENSION DUE TO RENAL ISCHAEMIA

Rise in blood pressure is obtained when ischaemia of either or both kidneys is produced. If only one kidney is involved the blood pressure usually increases less and more slowly than if the two are involved; moreover, after a time it has a tendency to fall gradually to the normal level [Loesch, 1933; Goldblatt *et al.* 1934; Elaut, 1936; Houssay & Fasciolo, 1937 *a*, *b*, etc.].

Ischaemia of the kidney is the cause of hypertension, since on removal of a unilaterally treated kidney the raised blood pressure rapidly falls to normal, as shown by Goldblatt *et al.* [1934], Dicker [1937 *a*], Houssay & Fasciolo [1937 *a*]. This is also shown by the fact that on removal of the forceps causing compression of the renal artery the blood pressure returns to normal [Dicker, 1937 *a*; Goldblatt, 1937].

The hypertension is not due to uraemia, since in many animals with high blood pressure the blood urea is normal. Also, when bilateral nephrectomy is performed, there is a fall in the arterial pressure 40 hr. later [Houssay & Taquini, 1938], a fact which is already well known.

ROLE OF THE NERVOUS SYSTEM

The hypertension is not caused by a reflex starting from the kidney, since it can be produced and maintained after large resections of the nervous vasoconstrictor system. The hypertension produced by renal ischaemia is not prevented or cured by renal denervation [Page, 1935; Collins, 1936; Goldblatt, 1937] or section of the splanchnic nerves even when associated with removal of the last four thoracic sympathetic ganglia [Goldblatt, Cross & Hanzal, 1937]. Neither is it cured by section of the anterior roots from the sixth dorsal to the second lumbar [Goldblatt & Wartman, 1937], nor by complete removal of the sympathetic chains [Freeman & Page, 1937; Heymans, Bouckaert, Elaut, Bayless & Samaan, 1937; Alpert, Alving & Grimson, 1937]. It is also not prevented by bilateral section of the splanchnic nerves and the sympathetic lumbar chains [Introzzi, Canonico & Taiana, 1938]. Because of these results Goldblatt [1937] states: "this type of experimental hypertension is due primarily to a humoral and not to a nervous mechanism initiated by the ischaemia of the kidney."

KIDNEY EXTRACTS

Unsatisfactory results have been obtained by efforts to demonstrate the existence of a substance producing renal hypertension in extracts of ischaemic kidneys; besides it would be more interesting to know what is produced and excreted into the blood than what is stored in the parenchyma.

Tigerstedt [1897] and Tigerstedt & Bergmann [1898*a*, *b*] have pointed out that extracts of normal kidney have a hypertension producing action, and this has been confirmed by others. Recently two groups of investigators, Harrison, Blalock & Mason [1936], on the one hand, and Prinzmetal & Friedman [1936] on the other, have made a comparative study of the action of normal kidney extracts and extracts of ischaemic kidneys. Extract of normal dog's kidney, when injected intravenously in dogs, produces an initial drop in arterial blood pressure followed by a gradual and prolonged rise which lasts 10–30 min. or more. Extract of ischaemic kidney produces usually less initial drop and a larger secondary increase than that of normal kidney. Prinzmetal & Friedman say that these results are compatible with the theory that the hypertension can be due to an excess of a blood pressure raising substance normally present in the kidney, but that the data are not sufficient to establish a causal relationship between the blood pressure raising substance of the kidney and the arterial blood pressure. Recently Harrison, Blalock, Mason & Williams [1937] have tried the extracts on rats anaesthetized with pentobarbital because these animals do not react with the initial hypotension. Extracts of kidneys from six dogs with hypertension caused a larger increase in blood pressure than extracts of kidneys from five normal dogs.

ISCHAEMIC KIDNEY GRAFT

Houssay & Fasciolo [1937*a*] grafted an ischaemic kidney in the neck of a chloralosed dog, uniting the artery to the carotid and the renal vein to the jugular; this was done by means of Payr's canulae, one of which connected the carotid with the aortic opening of the renal artery and the other connected the jugular with the renal vein (Fig. 1). The dog which received the graft was previously nephrectomized and its arterial blood pressure recorded during 2 hr. At the end of this time the donor was anaesthetized with ether or chloralose and the kidneys removed and then grafted into the receptor, the operation being completed in from 5 to 15 min.

Using this technique twenty-four kidneys in which the renal arteries had been compressed from 3 to 40 days, provided by twenty-four dogs with raised blood pressure (between 175 and 210 mm. of mercury), were grafted into thirty receptors.

When the blood flowed from the carotid through the graft and was seen to return by the renal vein, the blood pressure rose rapidly, rising by 32–70 mm. Hg in 5–10 min. Later it remained stable at this figure or slightly lower (Figs. 2 and 3).

If the grafted kidney was removed and placed in the neck of another dog, this in its turn had a rise in blood pressure; meanwhile the blood pressure of the first dog fell slightly, but sometimes not at all, during the first 20–30 min., and remained high for several hours. When the kidney was regrafted a second time into the first dog the blood pressure increased little or not at all when it was already high due to the first grafting.

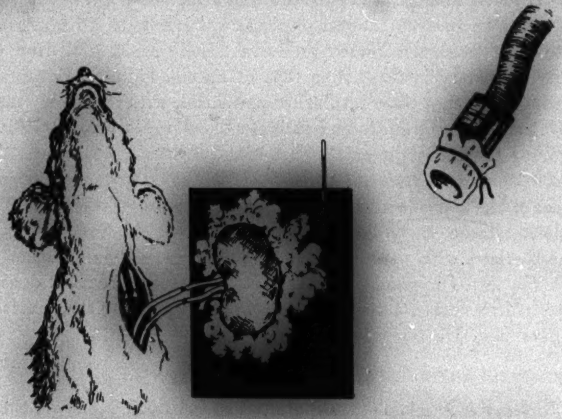

Fig. 1. Kidney grafted into the neck by arterial and venous anastomosis. Above, a vessel with a Payr's canula.

In the graph of Fig. 4 the rise in blood pressure obtained in sixteen out of seventeen dogs is shown. Such a rise was seen in twenty-seven of the thirty grafted dogs. In two of the three negative experiments the kidneys were each grafted with good result in one dog, in the third the ischaemic kidney did not cause a rise of blood pressure in a resistant dog, but did so when grafted in another animal.

In the same way twenty-five kidneys obtained from twenty-two normal dogs were grafted into the necks of twenty-seven chloralosed, recently nephrectomized dogs (Fig. 5). The kidneys from nineteen dogs did not produce any rise in blood pressure after grafting. The kidneys of three dogs produced rises of 20, 39, 45 and 70 mm. Hg. In the last experiment one kidney produced a significant rise of blood pressure in two dogs, the blood pressure rising in one from 125 to 195 and in the other from 140 to 185 mm. Hg. Unfortunately there was no histological examination to prove whether or not the kidneys were really healthy. In three cases the normal kidneys were removed and left between 30 and 45 min. at room temperature and then regrafted, but even so there was no rise in blood pressure.

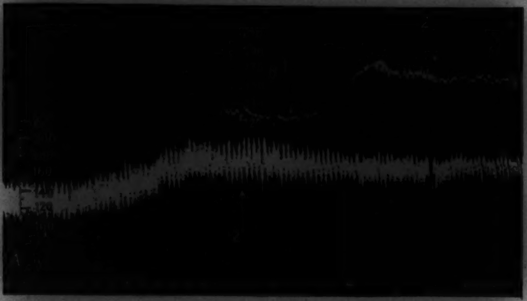

Fig. 2. Graft of an ischaemic kidney from a dog with high blood pressure (200 mm. Hg) into two nephrectomized chloralosed dogs. A, Dog. 12·5 kg. 1, graft placed in neck; 2, graft withdrawn. B, Dog 11·0 kg. 1, graft placed in neck; 2, graft withdrawn.

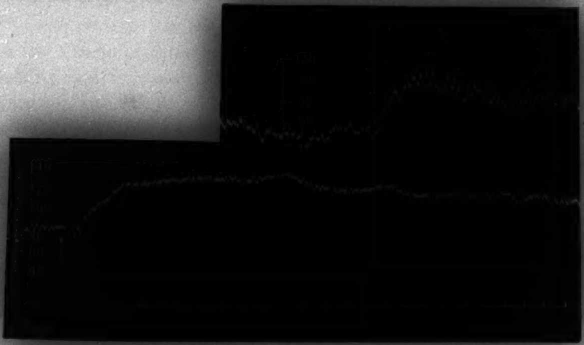

Fig. 3. Similar experiment to that of Fig. 2. Ischaemic kidney from a dog with raised blood pressure; the arrows mark when it was grafted into the neck, first in one and then in another chloralosed nephrectomized dog.

Comparative grafts of the ischaemic and healthy kidneys of animals operated on one side only were performed. While the ischaemic kidney always produced a rise in blood pressure the healthy kidney usually did not. Nevertheless, in one case the healthy kidney produced a rise in blood pressure which presently became stable; when this occurred the kidney was

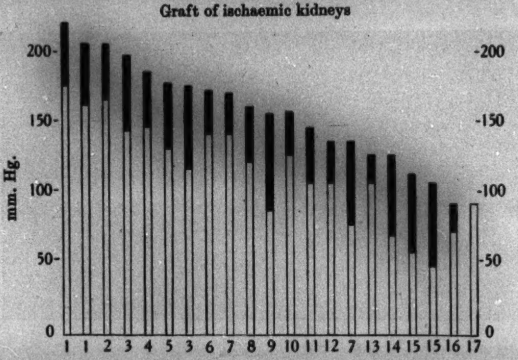

Fig. 4. Each column is an experiment with an ischaemic kidney from a dog with raised blood pressure (180–210 mm. Hg) grafted into the neck of a chloralosed nephrectomized dog. The white column indicates the initial level of the blood pressure, the black the increase produced by the graft. The lower numbers correspond to the record numbers of the dogs.

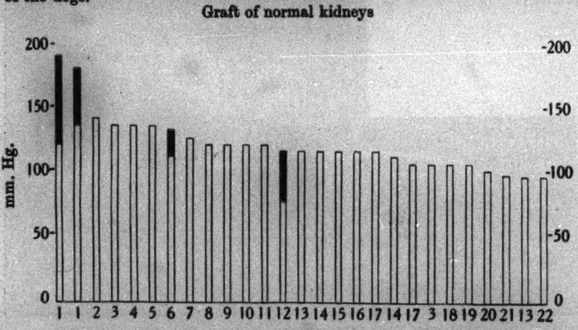

Fig. 5. Each column represents an experiment with the graft of a normal kidney into the neck of a chloralosed nephrectomized dog. The white column indicates the initial blood pressure, the black the increase produced by the graft. The lower numbers correspond to the record numbers of the dogs.

removed and the ischaemic kidney grafted instead, producing a further rise in blood pressure; this seems to demonstrate that the ischaemic kidney produces a more active substance than the healthy one.

In one case the kidney had two renal arteries, one of which was compressed. This kidney was grafted by anastomosing first the untouched artery; this did not produce a rise in blood pressure. The kidney was then connected by the compressed artery, and this produced a rise in blood pressure. Each time the kidney was grafted it was possible to see perfectly marked out the areas supplied by each of the two arteries.

These experiments show that the ischaemic kidney induces hypertension in the dog by producing a secretion which causes a rise of the normal blood pressure till it reaches hypertension level and maintains it there.

Dicker [1937*b*] has confirmed the fact that a kidney grafted into the neck of a normal dog can cause a rise in arterial blood pressure.

The vasoconstrictor substance is produced by a secretion of the ischaemic kidney and not by an autolysis of the organ, since its action is maintained during months and at autopsy the organ may present good size and appearance.

ACTION OF THE VENOUS BLOOD OF THE ISCHAEMIC KIDNEY

Comparative studies have been made [Houssay & Taquini, 1938] on the vasoconstrictor action of the plasma of venous blood from both healthy and ischaemic kidneys. The blood of chloralosed animals was collected and mixed with an equal volume of Ringer's fluid with 4 % sodium citrate, it was rapidly centrifuged and the plasma diluted to a quarter strength with Ringer's fluid. In this way plasma, containing 0·5 % of citrate and diluted with calcium-free Ringer's fluid to eight times its original volume, was finally obtained.

The vasoconstrictor action of these plasmas was tested by Laewen-Trendelenburg's method in the toad *Bufo arenarum* Hensel. The efferent drops of the preparation were electrically registered together with the time in minutes. A special apparatus allowed the perfusion to take place at a constant pressure of 15–25 cm. of water, and the liquids were easily changed without modifying the pressure.

First, Ringer's solution was perfused for at least ½ hr., till a constant outflow was obtained, one plasma was then perfused for 20 min., then replaced by Ringer, and, finally, the other plasma was perfused. In one toad the venous blood from a normal kidney was first perfused and in another the venous blood from the ischaemic kidney of a dog with raised blood pressure.

The venous blood plasma from ischaemic kidney always had an intense vasoconstrictor action (Fig. 6) which began immediately, increased for several minutes and then remained steady (Fig. 7). When Ringer's fluid

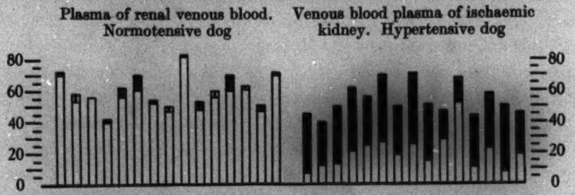

Fig. 6. Vasoconstrictor action of citrated blood plasma in a vascular preparation of a toad according to the Laewen-Trendelenburg method. Each column corresponds to one plasma; the height indicates the initial number of efferent drops from the preparation perfused with Ringer's solution. The decrease in drops is indicated by the black columns, the increase by the striped columns.

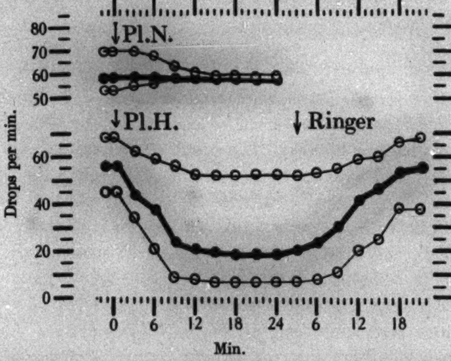

Fig. 7. Vascular preparations of toads according to the Laewen-Trendelenburg method. Number of drops per min. Pl.H., plasma from venous blood of ischaemic kidneys (high blood pressure); Pl.N., venous blood plasma from normal kidneys. The thin line is the largest and smallest constriction with each type of plasma. The thick line is the average action of the plasma from fifteen dogs.

was perfused later the vasoconstrictor effect gradually disappeared. The renal venous plasma from fifteen dogs with hypertension tried in twenty toads decreased the initial number of drops by 63 %, the maximum decrease being 84·5 % and the minimum 18·5 %.

The venous blood plasma from the normal kidney of fifteen dogs was little or not at all active. There was a slight decrease in the number of drops in nine cases, a slight increase in five and no alteration in one. The maximum decrease was 9·4 % and the average 2·8 %.

The vasoconstrictor action is not due to uraemia, it occurs with the renal plasma of dogs with hypertension in which the blood urea is normal.

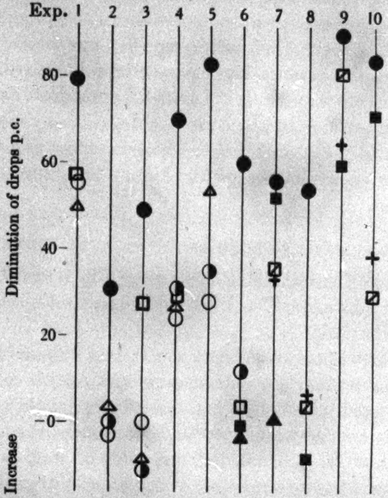

Fig. 8. Surviving vascular system of the toad *Bufo arenarum* Hensel (Laewen-Trendelenburg method). Percentage diminution of drops (vasoconstriction) or augmentation of drops (vasodilatation), produced by the plasmas (diluted 1 : 10 and citrated to 0·4 p.c.) from venous blood of different organs of hypertensive dogs. The hypertension was produced by renal ischaemia of the only remaining kidney. Each vertical line corresponds to one dog.

○ Vena jugularis pl. ◐ Carotis comm. pl. △ Lienal v. pl.
▲ Hind leg pl. ■ Hepatic v. pl. + Right heart pl.
▨ Left heart pl. ● Renal pl. □ Mesenteric v. pl.

It was also shown that 40 hr. after bilateral nephrectomy the blood plasma from the right heart of five uraemic dogs did not give a vasoconstrictor action.

These experiments show that the ischaemic kidney produces a substance with an intense direct vasoconstrictor action. The vasoconstrictor action is not seen with the ultrafiltrate of the citrated plasma using acetic collodion membranes nor when the plasma is deproteinized according to the method of Folin & Wu.

Houssay & Taquini [1938] have compared the action of venous blood plasma from the different organs of ten animals with hypertension of renal origin. The venous blood from the ischaemic kidney and from three to five different organs was collected, avoiding asphyxia and taking care to reduce reflexes to a minimum.

In all cases the venous blood plasma from the ischaemic kidney caused a higher vasoconstrictor action than any other blood (Fig. 8). The blood from the carotid, right and left hearts, splenic, mesenteric, hepatic and femoral veins have all been studied separately, and these experiments all show that the ischaemic kidney is the essential or only site for the production of a vasoconstrictor substance in the dog with hypertension. This substance accumulates in the blood to a certain extent and appears to be destroyed in certain organs, the healthy kidney being specially active, as will be seen later.

THE PROTECTING ROLE OF THE HEALTHY KIDNEY

Healthy renal tissue eliminates or destroys in a varying degree the vasoconstrictor substance. This has been shown by the various experiments of Fasciolo [1938*a*, *b*].

(1) Extirpation of one kidney does not produce a rise in blood pressure. If 8 or 10 days later the artery of the remaining kidney is compressed, the blood pressure rapidly rises and is maintained at a high level with oscillations. In animals which were observed for 6 months the blood pressure was maintained at 220–250 mm. Hg from the 40th day after the operation. If the renal artery of one kidney is compressed, the other remaining intact, the rise in blood pressure is less rapid and is not always maintained. In most cases the blood pressure returns to normal.

In a series of eighteen dogs having a single ischaemic kidney there was an average rise in blood pressure of 11·7 mm. Hg per day, while in a series of twenty-three dogs with one ischaemic kidney and the other intact, the average rise was 5·2 mm. Hg per day. In the dogs with the single ischaemic kidney the average rise was 47 mm. at the 4th day, while in those which had a healthy kidney as well the average rise was only 37 mm. in 7 days.

(2) If the healthy kidney in the dogs with one ischaemic kidney is removed when the blood pressure is stable or declining, the blood pressure rapidly rises to a high level and is maintained there. The average increase was 28 mm. for eight dogs 7 days after the one kidney had been made ischaemic, at the 17th day the increase had declined to 18 mm. The healthy kidney was then removed, and in 8 days only the increase was 60 mm. Hg, giving a blood pressure of 200 mm.

(3) Grafting of the ischaemic kidney of a dog with hypertension in the neck of another dog caused a greater increase in nephrectomized animals than in those which had their kidneys. The average rise in seven comparative experiments was 32 mm. Hg in the nephrectomized animals and 4 mm. in animals with kidneys intact.

(4) When on one side the kidney has been made ischaemic, its venous blood has a potent vasoconstrictor action, which is greater than that of the general blood stream. On the other hand, the blood which leaves the healthy kidney can show a marked reduction of this action compared with the blood which supplies it.

All these experiments show that the normal kidney is capable of reducing the rise in blood pressure produced by the ischaemic kidney.

ROLE OF THE ENDOCRINE GLANDS IN HYPERTENSION OF RENAL ORIGIN

Page & Sweet [1937] have observed that the rise in blood pressure due to renal ischaemia can occur, but is not maintained, in absence of the hypophysis. We have obtained increases to 170 and 195 mm. Hg starting from 120–140 mm. According to Page & Sweet the rise is produced but is less marked and transitory, particularly if there is adiposity or a low basal metabolism. Hypophysectomy carried out in dogs with hypertension causes a reduction of the blood pressure to the normal level or slightly higher than normal in 20 days. Page & Sweet say that "the effect of hypophysectomy on hypertensive dogs is believed to be an indirect one. It is postulated that the responsiveness of the blood vessels to chemical stimuli from the kidneys with constricted renal arteries is reduced. This may be due to lack of the secretions of the adrenal and thyroid glands. Deficiency of these secretions may in turn be due to withdrawal by hypophysectomy of the chemical stimuli normally afforded them by the hypophysis."

Thyroidectomy neither prevents nor cures the arterial hypertension due to ischaemia of the kidney [Glenn & Lascher, 1938].

Extirpation of the right adrenal and the left adrenal medulla, with or without section of the splanchnic nerves, does not prevent hypertension from being produced by the ischaemic kidney [Goldblatt *et al.* 1934; Fasciolo, 1938*a*, *b*]. It is only necessary to leave a small piece of adrenal cortex for the blood pressure to rise and remain high [Goldblatt, 1937].

The role of the adrenal has been studied by Goldblatt [1937]. Bilateral extirpation of the gland prevents the rise in blood pressure or causes it to drop if it has already risen. This has been observed also in various animals kept alive for several months with sodium salt treatment. Nevertheless, in two cases the blood pressure rose under these conditions. Extract of the cortex caused the blood pressure to rise.

Introzzi *et al.* [1938] have observed that the blood pressure in dogs with hypertension drops, sometimes to normal, after resection of the splanchnic nerves and the lumbar sympathetic chains and a large part of the adrenals, but these observations only lasted for 40–60 days.

Fig. 9. The arrow indicates the grafting of the ischaemic kidney from two hypertensive dogs into the neck of two adrenalectomized and nephrectomized dogs.

Fasciolo [1938*a*, *b*] has shown that grafting the ischaemic kidney of a dog with hypertension produces the same rise in blood pressure in chloralosed nephrectomized dogs whether they have adrenals or not. The average rise in six comparative experiments was 26–30 mm. Hg respectively. In the graph (Fig. 9) it can be clearly seen that the vasoconstrictor substance secreted by the grafted ischaemic kidney produced its blood pressure raising effect independently of the adrenals.

The right adrenal was removed in chloralosed dogs and the adrenal veins temporarily compressed; an ischaemic kidney from a dog with hypertension was grafted into the neck and the blood pressure rose in three cases. When the blood pressure remained stable the forceps were removed from the adrenal veins, but the blood pressure did not rise [Fasciolo, 1938*a*, *b*]. Therefore the substance causing hypertension produced by the ischaemic kidney does not act through the adrenal.

The results obtained by Goldblatt can probably be explained because cortin favours the production of hypertension either by increasing the formation of the vasoconstrictor substance by the kidney or perhaps by increasing the sensitivity of the vasomotor system to its action.

For reasons of space the visible and histological changes observed in the animals with hypertension as well as other interesting points have not been discussed.

SUMMARY

Evidence is presented that the ischaemic kidney secretes directly a vasoconstrictor substance which causes a permanent hypertension. This substance is active in the absence of the adrenals. The healthy kidney is capable of diminishing the action of this blood-pressure raising substance.

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REFLEXOGENIC AND CENTRAL STRUCTURES IN OXYGEN POISONING[1]

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SINCE the work of Bert [1878] it has been recognized that oxygen at high barometric pressure exerts acute deleterious effects on animal organisms. The entire story as to just how this oxygen poisoning is brought about is not yet known. There can be little doubt, however, that one of the most important factors is the upset in the carriage of carbon dioxide, which results from the failure in the reduction of oxyhaemoglobin at the tissues when an animal breathes pure oxygen at pressures greater than 3 atm. This explanation was first suggested some years ago by Gesell [1923], who had observed that the susceptibility of rats to high oxygen was increased when a small amount of carbon dioxide was added to the respired oxygen. A more direct attack of this problem was made later [Bean, 1929] in experiments in which dogs were exposed to pure oxygen at pressures of from 3 to 5 atm. Oxygen pressures above 3 atm. (the point at which there would theoretically be a failure of the normal reduction of haemoglobin) caused an increased blood acidity, a decreased heart rate and a lowered oxygen consumption, all of which support the contention of haemoglobin involvement. Moreover, shortly thereafter Campbell [1929] found an increased carbon dioxide pressure, high enough to be toxic, in the tissues of rabbits breathing pure oxygen at 3½ atm. That there is a failure of normal reduction of haemoglobin under high oxygen was demonstrated in experiments in which blood colour changes were followed continuously in dogs [Bean, 1931]. Experiments of Hill [1933] have indicated the importance of carbon dioxide in oxygen poisoning. Another group of investigators who had concluded [Behnke, Shaw, Shilling, Thomson & Messer, 1934] that carbon dioxide played no significant role in oxygen poisoning later reversed their opinion and state: "Carbon dioxide tensions which are wholly innocuous when associated with oxygen pressures of less than 1 atm., prove highly toxic when associated with oxygen at 4 atm. of pressure" [Shaw, Behnke & Messer, 1934]. It is quite possible there are other contributing factors to the production of oxygen poisoning. Recent experiments of Campbell [1937] indicate that the thyroid gland and external temperature play important roles, and apparently high oxygen also has some direct toxic influence on isolated frog muscle [Bean & Bohr, 1938].

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The site of action of the accumulated carbon dioxide, increased acidity and perhaps additional factors in the production of the convulsive seizures of oxygen poisoning has been little more than a subject for speculation. Conceivably several structures might be either contributory to, or perhaps entirely responsible for, such seizures in animals exposed to oxygen at high barometric pressures. In the present study we have attempted to determine the importance of some of these structures which might be peculiarly involved in the induction of these convulsive attacks.

Apparatus and methods

The chamber in which the experimental animals were exposed to high pressure was essentially that, with few modifications, which has been described earlier [Bean, 1931; Bean & Haldi, 1932]. This chamber permits the continuous recording of respiration, blood pressure and heart rate, changes of blood pH and volume flow on smoked paper of a long-roll kymograph. A glass window provides for direct continuous observation of the animal which is at all times completely under the control of the operator—as much as though the operator were himself present in the chamber. The temperature may be regulated at will. Intravenous injection may be controlled simply by the manipulation of two stopcocks. Continuous observation of colour changes in the circulating blood and the sampling of circulating arterial or venous blood are easily accomplished. Uni- or bilateral blockage of the vagus nerves is attained by circulating cooled solution from the outside through tubular hooks in which the nerves have been previously slung. These hooks are insulated from the surrounding tissue in which they are buried. Removal of the cold blocks—referred to below as deblocking—is attained simply by stopping the circulation of the cooled fluid.

Given such regulating devices as our apparatus affords, we cannot subscribe to the views of some investigators [Behnke *et al.* 1934] concerning experiments in which the pressure chambers have been of such size as to prohibit the entrance of the operator. Not only have we found the chamber employed in our present and earlier investigations admirably suited to the various problems undertaken, but we also recognize obvious advantages, such as greater ease of compression, temperature regulation and the economy accruing from the use of such a chamber for animal experiments. More important, however, is the equally obvious fact that such apparatus eliminates the quite unnecessary risk to the operator of the long recognized deleterious effects of high oxygen pressure. In many experiments on high oxygen pressure, data may lose their objectivity or are distorted by the subjective effects of the high oxygen pressure where the operators are themselves exposed along with the experimental animal. That high oxygen pressure and even high air pressure make the exposed operator incapable of properly recording or observing objectively is clearly brought out by these same advocates of large chambers: Under 10 atm. of air... "A simple task, the palpation of the pulse of another worker was accomplished only with extreme difficulty" [Behnke, Thomson & Motley, 1935]; and from an earlier report, a human subject under 4 atm., "after breathing oxygen for 43 min.... suddenly experienced a transient syncope associated with a blanching of the face, sweating of the hands and absence of the radial pulse" [Behnke, Johnson, Poppen & Motley, 1934*a*]. Damant [1930], and Hill & Phillips [1932] report subjective effects and altered cerebration as a result of exposure to increased pressure. It is apparent then that wherever the investigations on high oxygen pressure permit, the regulation of the experiment and the observations should be made by an operator from without the compression chamber, regardless of its size.

The experimental animals used in our present study were dogs, partially or totally decerebrated. Such preparation served a twofold purpose, viz. (1) helping to determine whether convulsive seizures induced by high oxygen pressure can occur in the absence of the cerebral cortex and lower nuclei of the cerebrum, (2) eliminating the use of any prolonged chemical anaesthesia and thereby also the possibility that any reaction which might occur during or subsequent to exposure to high oxygen pressure could be due to pain attendant upon a lowered chemical anaesthesia. This last-mentioned point is of considerable significance, since it has been found that high oxygen pressure rapidly lowers chemically induced anaesthesia [Bean, 1931] to such degree that the movement and reaction of the animal can very easily be misinterpreted as oxygen poisoning.

The decortication and decerebration were carried out under a number of different transient anaesthetics, morphine urethane, Evipal or ether. Except for those few experiments in which the thalamus was left intact the cerebrum was removed down to the level of the superior colliculi.

After sufficient time (usually 1–1½ hr.) had elapsed for the transient anaesthesia to wear off, the animal was exposed in the chamber to oxygen at from 3 to 5 atm. pressure. Blood pressure, heart rate, and respiration were recorded and the animal's behaviour closely observed.

RESULTS AND DISCUSSION

The onset of oxygen poisoning was, with very few exceptions, first manifest by an alteration in breathing and frequently by twitching of some facial or neck muscle. There was usually an early hyperpnoea, jerky and irregular respiratory movements which later changed to a slowed deepened dyspnoeic breathing. The expiratory effort became progressively more pronounced and prolonged. Not infrequently apnoeic periods appeared in the expiratory phase, some as long as 25 min. have been recorded and observed; pronounced periodic breathing was not an uncommon reaction [Bean, 1932]. Tonic convulsive expiratory movements involving the greater part of the body musculature occurred. The convulsive seizures frequently spread also into the inspiratory phase. In addition to these manifestations of oxygen poisoning, tonic and clonic movements of the limbs unrelated to the respiratory cycle, dilatation of the pupil, emesis, excessive salivation, relaxation of the sphincters with resulting defaecation and urination, have been commonly observed.

While severe convulsive attacks may appear suddenly, the oxygen poisoning is a gradual process beginning with the failure in the reduction of oxyhaemoglobin. That there is wide individual variation in the susceptibility to high oxygen pressure is explained in part at least by a correspondingly wide individual variation in the response to carbon dioxide. For these reasons it would appear dangerous to set any time limit of exposure for which an animal can be assured of safety from oxygen poisoning.

The cerebrum and oxygen poisoning. Three experiments were performed on animals, from each of which the cerebrum had been removed down to the thalamus. Exposure of these animals to 5 atm. of oxygen resulted in convulsive seizures typical of those described as oxygen convulsions. Twelve other animals, in which decerebration had been carried to the level of the superior colliculi, reacted to high oxygen pressure in an essentially similar manner.

Reflexogenic zones and oxygen poisoning. Since the onset of oxygen convulsions is first manifest by an upset of breathing, and since also the seizures in the more advanced stages are so intimately associated with respiratory movements, a consideration of causative or contributory mechanisms must include those reflexogenic zones which play important roles in the control of breathing, viz. the carotid sinuses and bodies, the aortic and pulmonary sensory areas served by the vagi.

Bilateral denervation of the carotid sinuses and bodies was performed on ten decerebrate dogs. This procedure, like decerebration alone, did not prevent the occurrence of either the disturbances in breathing which precede the actual convulsive seizures or the convulsive seizures themselves. Considering the individual variations in the reactions of animals to high oxygen pressure these sinus denervation experiments clearly indicate that the functioning of the carotid sinus or carotid body receptors is not essential to the occurrence of either the early respiratory disturbances or the convulsions. It is recognized nevertheless that these structures with innervation intact cannot be dismissed as being unaffected by high oxygen pressure. Indeed, one might well expect that in exposure to high oxygen pressure the resulting accumulation of carbon dioxide, increased acidity and lowered oxidation would bring about some stimulation of the chemoreceptors of the carotid bodies with consequential changes in cardiac, respiratory and vasomotor activity.

In non-decerebrate animals with intact sinuses and anaesthetized with morphine urethane, exposure to high oxygen pressure causes a slowing of the heart [Bean, 1931]. In our present experiments this bradycardia was found to be much less pronounced in decerebrate sinus denervated animals. Apparently, therefore, the intact sinus mechanism does contribute to the production of this slowing. However, the fact that a high oxygen bradycardia occurs in the denervated sinus preparations indicates involvement of additional cardiac control mechanisms.

The onset of severe oxygen convulsive seizures was attended by a sharply increased heart rate superimposed upon the bradycardia just mentioned. This tachycardia has been recorded from animals not only with intact, but also with denervated sinuses. It cannot be attributed, therefore, to increased carotid sinus or carotid body activity. The more probable explanation is either a gross inadequacy of the sinuses and bodies to meet, even at their highest state of efficiency, overwhelming demands, or an actual decrease or complete paralysis of function of the sinus and carotid body receptors or their central connexions by accumulated carbon dioxide and acidity.

Vagi and oxygen poisoning. The vagal trunks mediate afferent impulses from aortic and pulmonary endings which play important roles in reflex regulation of breathing and circulation. Does high oxygen pressure induce the respiratory convulsions of oxygen poisoning by action on these endings? Investigation of the possible role of the vagi in oxygen poisoning was begun with preliminary experiments on non-decerebrate rats anaesthetized with morphine and urethane. In each of such experiments two litter mates were used; in one of these the vagi were sectioned; the other animal was used as control. Although the early experiments presented no conclusive results, due in part to the depth of anaesthesia required, they did suggest that the intact vagi may function in a protective manner to delay the onset of oxygen convulsions. In our later experiments on dogs no attempts were made to isolate either the aortic or the pulmonary branches of the vagi, so the corresponding reflexogenic zones were considered together although they may act quite independently. In several experiments decerebrate dogs were vagotomized by complete transection of both vagi, but in most of the experiments on the vagi cold block (2–5° C.) was employed.

Cold blockage of the vagus nerves of decerebrate dogs, applied before the onset of the symptoms of oxygen poisoning failed to prevent, and cold blocking or sectioning these nerves after the onset of poisoning failed to relieve, the poisoning reactions already manifest. In fact, such vagal treatment usually hastened the onset and exalted the poisoning reactions. Vagal cold block applied before raising the oxygen pressure or in the early part of the period of exposure to the increased pressure established a slowed deepened dyspnoeic breathing with convulsive expiratory movements similar to and in many instances identical with that mentioned above which occur in the more advanced stages of oxygen poisoning. This effect of vagal cold blockage at atmospheric pressure is illustrated in Fig. 1, part 1. The animal in this experiment had been decerebrated under transient ether anaesthesia, and, as was typical of such, was more responsive to experimental procedures than were those decerebrated under transient Evipal anaesthesia.

Vagal cold block applied after some time of exposure to high oxygen pressure, but before the onset of the convulsive seizures, induced the convulsive respiration which frequently continued after deblocking, apparently maintained by the oxygen effect itself. Such vagal block effect of high oxygen pressure was relieved by decompression to atmospheric pressure or by substituting air at an equally high pressure, for the oxygen. Such results are illustrated in Fig. 1 A, B, C, D, the records of which were obtained from an experiment in which Evipal had been used during the decerebration. Part A shows the effect of cold blocking and deblocking the vagi while the animal breathed oxygen at atmospheric pressure. In contrast to the blockage response in animals decerebrated under ether there was no dyspnoeic prolonged expiratory phase and no convulsive movements. Vagal cold block applied to this animal after it had breathed oxygen at 60 lb. gauge pressure for 25 min. gave rise to the record shown in part B. This response more nearly approximated that observed in the animals decerebrated under ether (Fig. 1, part 1), in that the prolonged dyspnoeic expiratory phase with convulsive muscular movements was present. The vagal blockage effect persisted for some time after deblocking and there followed an apnoeic period of 12 min. (not shown in the figure) before the return to the preblock type of breathing as seen in part C. High oxygen pressure had evidently increased the response to vagal cold blockage. After the exposure to high oxygen pressure had been continued for 32 min. more (87 in all), the breathing, as shown in part D, had become practically identical with that of part B. Not only are the records B and D identical, but also the accompanying convulsive muscular movements were essentially similar. High oxygen pressure acting alone in this instance (part D) had induced reactions indistinguishable from those previously induced by cold blocking the vagi after a short exposure to high oxygen pressure (part B). It is noteworthy also that this reaction (Fig. 1, part D) was very similar to that produced by vagal cold block alone, applied at atmospheric pressure in dogs decerebrated under ether. After the return of the animal to

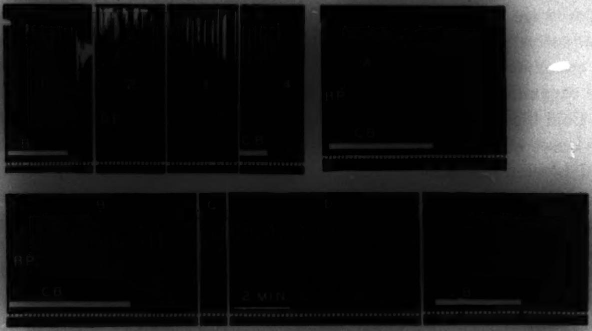

Fig. 1. High oxygen effects and vagal cold blockage: 1, 2, 3, 4, dog decerebrated under *transient* ether anaesthesia; A, B, C, D, decerebration under transient Evipal anaesthesia. In each, from above downward, respiration (upstroke = Expiration) blood pressure (*B.P.*), Signal (*C.B.* = cold block applied to vagi), time (2 min. = 2 minutes). For more complete explanation see text.

atmospheric pressure (Fig. 1, part E) the response to vagal cold blockage approximated that which occurred before compression. The results of this experiment indicate that high oxygen pressure enhances the effects of vagal cold blocking; and that high oxygen pressure acting alone may eventually induce vagal blockage effects, in all probability by blocking out vagal influences on central mechanisms.

Vagal cold blockage and high oxygen pressure act in a similar manner on animals prepared under ether anaesthesia. Illustrative of this are the records shown in Fig. 2: A, the effect of vagal cold blockage while breathing oxygen at atmospheric pressure before compression; B, the response to vagal cold blocking and deblocking after breathing oxygen at 60 lb. pressure for 70 min.; C, the respiratory record after 20 min. more exposure to oxygen at 60 lb. when the high oxygen pressure of itself had induced a response essentially similar to that induced earlier by the vagal cold block. Application of the cold block at this stage (C) was without any further significant effect, the oxygen having already given rise to a maximum blocking action and the attendant convulsive expiratory movements. Rapid relief from this convulsive breathing and the accompanying tonic and clonic body movements was attained by replacing the spirometer oxygen with room air at the existing chamber pressure (60 lb.). This is shown in Fig. 2, part D. Vagal cold block applied during the breathing of air at 60 lb. pressure was, in contrast to that during high oxygen pressure or before compression, remarkably ineffective (Fig. 2, part E). The subsequent shift of the animal back to breathing high oxygen pressure (not shown in figure) was attended by a return of the effectiveness of vagal cold blocking and also the return of the vagal block effect of the high oxygen pressure itself, similar to that

Fig. 2. Increasing reaction to vagal cold blockage as high oxygen exposure is prolonged; onset of convulsive type of breathing and vagal blockage effect of high oxygen; reversal of effects by substituting room air at high pressure for the high oxygen. From above downward, respiration (upstroke = Expiration), Signal (*C.B.* = cold block applied to vagi), time (2 min. = 2 minutes duration). The long displacement of the respiratory record in D is an artefact due to an additional rinsing of the spirometer with room air at high pressure. See text.

shown in parts B and C. Decompression to oxygen at atmospheric pressure resulted in the disappearance of the signs of oxygen poisoning, but the sensitivity of the vagi to blocking fell to a much lower level than that prevailing previous to exposure to the high oxygen exposure (part A).

The rapidity of recovery from the vagal block effects and convulsive manifestations of high oxygen poisoning, when the oxygen was replaced by room air at an equivalent pressure, was not dependent upon the integrity of the carotid sinuses or bodies. Evidence for this is shown in Fig. 3, which was recorded from a decerebrate denervated sinus animal. Here convulsive movements and the vagal block effect of high oxygen pressure (part D) were relieved by a shift to room air, and this, even before the change to air which occupied a period of less than 3 min., was completed. Apparently the causative agent in oxygen poisoning was effective on central structures aside from any influence it may have had on reflexogenic zones.

The vagi and bradycardia of oxygen poisoning. With the purpose of investigating the relationship of the vagi to the bradycardia of oxygen poisoning which occurs in decerebrate animals with intact or denervated sinuses and in non-decerebrate anaesthetized animals as mentioned above, several experiments were carried out in which the vagi were sectioned previous to exposure to the high oxygen pressure. It was found that the tachycardia resulting from such vagal sectioning was not significantly altered by the subsequent exposure to high oxygen pressure. Evidently the high oxygen bradycardia is mediated by the vagi, and any slowing influence which may be exerted by the oxygen directly on the heart or through the sympathetic innervation may be completely masked by the tachycardia of vagal sectioning.

The tachycardia which occurred so frequently with the onset of severe convulsive seizures and which was superimposed upon a heart previously slowed by high oxygen, might possibly be explained as due to a secondary blocking of efferent vagal fibres. In this connexion it is noteworthy that Hill & Macleod [1903] observed in the frog's heart that the vagus nerve endings "appear to be paralysed by exposure to high oxygen". It seems likely, therefore, that high oxygen pressure can produce a central vagal block and thus alter breathing; that it can initially slow the heart by efferent vagal impulses and secondarily increase heart rate by blocking the inhibitory action of the vagi.

In these and earlier experiments [Bean, 1931] on high oxygen pressure, it was found that unless there occurred muscular spasm and convulsive seizures, the changes in blood pressure were not at all pronounced. The more usual change when such did appear was a slight gradual rise. The records in Fig. 1 show the blood-pressure changes in an experiment performed on a decerebrate dog with carotid sinus innervation and vagi intact. Here, after breathing oxygen at 60 lb. pressure for 45 min. (part 2), the blood pressure had altered only slightly. Five minutes later, however, with the onset of severe convulsive seizures and tachycardia the blood pressure increased sharply (part 3). If a vagal block action of high oxygen was in any way responsible for this change, such blocking action had not reached a maximum since application of the cold blocks to the vagi resulted in a still further increase in blood pressure and respiratory convulsive movements (part 4).

A similar blood-pressure response to high oxygen pressure occurs in decerebrate animals after denervation of the carotid sinuses and bodies.

Fig. 3. Denervated sinus, decerebrate dog; A, breathing oxygen at atmospheric pressure; B, 5 min. after compression to 5 atm. of oxygen; C, 30 min. after compression; D, 58 min. after compression when convulsions had begun. At *X* the oxygen was replaced by room air at 5 atm. (the very wide swings of the respiration record are due to spirometer rinsing). Part E was recorded after having returned from breathing air at 5 atm. to oxygen at the same pressure.

This is illustrated in Fig. 3. The blood pressure, after the animal had been breathing oxygen at 60 lb. pressure for 50 min., increased by only 3 mm. Hg (part C). With the onset of convulsive seizures, the blood pressure rose appreciably (part D). Replacement of the oxygen by room air at 60 lb. pressure dispelled the convulsive movements and turned the rising blood pressure downward.

If high oxygen pressure causes any generalized peripheral vasoconstriction, as is thought by some to be the case [Behnke, Forbes & Motley, 1935 *a*], experimental evidence [Behnke *et al.* 1935 *a*; Bean, 1931] indicates the brain is exempt from vasoconstriction. The experiments of Hill [1908] on the bat's wing and the frog's foot showed that at air pressures as high as 70 atm. (equivalent to 14 atm. of oxygen) there was no observable change in the peripheral blood vessels. Aside from the question of vasocontriction, however, there can be little doubt but that the muscle spasm and tachycardia are contributing factors in the sharp rise in blood pressure which takes place during convulsive attacks of oxygen poisoning.

It has been stated [Shaw *et al.* 1934] that oxygen poisoning in the anaesthetized dog first manifests itself by a drop in blood pressure and that this drop constitutes the criterion of oxygen poisoning. In later work on man it was found that there occurred no change in blood pressure as a result of exposure to high oxygen pressure [Behnke *et al.* 1934*a*]. A subsequent report [Behnke *et al.* 1935*a*] concludes that a rise in blood pressure is an early manifestation of oxygen poisoning. Although our earlier and present data indicate in general a rise in blood pressure under high oxygen pressure, they indicate definitely that blood pressure change is not a good criterion of oxygen poisoning. Our experimental results as shown in Fig. 1 are at variance with the results of experiments on dogs anaesthetized with sodium barbital in which a fall in blood pressure "is the constant and early sign of oxygen toxicity and always precedes the convulsive seizure" [Behnke *et al.* 1935*a*]. If one is looking for an early sign of oxygen toxicity, the respiratory record offers a more reliable indicator. Individual variation in susceptibility to oxygen poisoning is considerable, but rare indeed have been the exceptions which fail to show an early alteration in breathing, such as an hyperpnoea or dyspnoea, as a result of exposure to high oxygen pressure. The prompt increase in respiratory minute volume when our animals, either with intact or denervated sinuses or intact or sectioned vagi, were exposed to increased oxygen pressure bears this out.

The respiratory minute volume (expressed in c.c. per kg. body weight) of seventeen decerebrate dogs was measured in as many experiments. Fifteen of these showed a pronounced increase in respiratory minute volume within 5 min. or less after the oxygen pressure had been raised to 60 lb. One of the remaining two showed a delayed increase after an initial decrease which lasted 18 min. The other in which there was a decreasing minute volume preceding compression failed to show any increase in minute volume after compression, and died after 60 min. of exposure. Of these seventeen experiments, one group of six was carried out on dogs in which both the vagi and sinuses were left intact; a second group of six was carried out on dogs in which the carotid sinuses were denervated, but the vagi left intact; the third group of five experiments was performed on dogs with sinuses left intact, but the vagi either sectioned or cold blocked. The first group, i.e. those animals with both vagi and sinuses intact, showed the most marked increase in respiratory minute volume as a result of exposure to high oxygen pressure. The second group, those animals with intact vagi and denervated sinuses, showed much less increase in respiratory minute volume on compression than the first group, but somewhat more than the third group in which the vagi had been sectioned and the sinuses left intact. The two animals which failed to show an immediate minute volume increase on compression belonged to this last group. These results indicate that the receptors of the aortic bodies and the carotid sinuses and bodies, contribute to the early increase in respiratory minute volume of oxygen poisioning.

Oxygen poisoning is in large measure a reversible effect. It should be emphasized, however, that decompression to atmospheric pressure after prolonged exposure to high oxygen pressure may be attended with considerable difficulty even when sufficient time is allowed to prevent bubble formation. Decompression to atmospheric pressure on several occasions resulted in a failure of respiration which could be relieved by partial recompression. It is possible that this respiratory failure may have been due to an alteration in membranes and their permeability to oxygen at the lung. It may also have been due to some irreversible alteration in the cells of the regulatory mechanism, as a result of prolonged high concentration of carbon dioxide, increased acidity or perhaps some deleterious effect of oxygen itself.

SUMMARY

1. Decorticate and decerebrate dogs were exposed to oxygen at from 5 to 6 atm. pressure in a chamber equipped so that observations and experimental procedures could be carried out with the animal continuously and completely under the control of the operator from without.

2. Neither the cortex nor basal ganglia are essential to the induction of oxygen poisoning reactions which must arise from sites below the superior colliculi.

3. While the carotid sinuses and bodies, the aortic and the pulmonary vagal sensory endings contribute to oxygen poisoning by increasing the respiratory minute volume, they are not essential to the occurrence of oxygen convulsions.

4. The results suggest that high oxygen pressure enhanced the effectiveness of vagal cold block and acts to paralyse the normal vagal influence on the controlling mechanism of respiration.

5. The bradycardia of oxygen poisoning is largely dependent upon the integrity of vagal-cardiac fibres. The tachycardia which frequently

accompanies the convulsions is due in part to a depressing or paralysing action of accumulated carbon dioxide, acidity or direct toxicity of oxygen on nerve connexions of the sinus region.

6. Although there usually is a gradual rise in blood pressure, with a sharp increase during convulsive seizure, blood-pressure change is not a good criterion of the onset of oxygen poisoning. The earliest indication of poisoning effects is usually an alteration in breathing.

7. Since there is a wide individual variation in susceptibility to oxygen poisoning it appears unwise to set any specific time limit for safety from oxygen poisoning.

8. The results of these experiments are entirely compatible with the view that the accumulation of carbon dioxide and increasing acidity of the tissues consequent upon a failure in reduction of haemoglobin and a resulting upset in the carriage of carbon dioxide by the blood is one of its most important causative factors in oxygen poisoning. It is recognized, however, that there may be other and more direct toxic effects of high oxygen pressure on the tissues.

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EARLY LESIONS OF VITAMIN A DEFICIENCY

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It is well known that in rats suffering from advanced avitaminosis A manifold infective conditions are present, but it is not so clearly realized that an animal may be in a severely pathological condition before it shows any marked outward symptom of vitamin A deficiency. A previous communication from this Institute [Richards & Simpson, 1934] laid emphasis not only on the variability in pathological condition that may be encountered in experimental animals after exactly similar treatment, but also on the early occurrence of pathological symptoms. Such conditions as pyelitis, tongue abscess, inflammation and ulceration of stomach and caecum, and keratosis of bladder and stomach were noted in many animals which had died or been killed very shortly after the first indication of a fall in weight. It seemed evident in such cases that disease must have been well established while the animal was still increasing in weight, and showing no obvious symptom of vitamin A deficiency. This was confirmed by the macroscopic detection of gastro-intestinal lesions in rats which had been only 3–5 weeks on the deficient diet. The early occurrence of night blindness in vitamin A deficiency in domestic animals, which has been noted by Hart & Guilbert [1937], shows that this is also true of other species. It seemed of importance therefore to determine at what stage of the avitaminosis evidence of degeneration in the tissues of rats could be detected histologically.

In recent years Mellanby and others have drawn attention to the occurrence of nerve lesions in animals receiving a diet deficient in vitamin A. Inco-ordination and paralysis have been observed in dogs, rabbits, pigs, rats and chicks, and in several cases degeneration has been demonstrated in the central and peripheral nervous systems. Mellanby [1934] has suggested that there may be an interrelation between the two types of sequelae which follow a deficiency of vitamin A in the diet, viz. loca infection and nerve degeneration, and has put forward the hypothesis that loss of neurotrophic control may be the primary cause of the infective lesions. The clinical symptoms of the nerve lesions in rats have been well described by Aberle [1934], and by Sutton, Setterfield & Krauss [1934], who give a good review of the literature. It is to be noted, however, that reports on the occurrence of these nerve lesions are still remarkably few in number considering the large volume of work that has been published on the subject of avitaminosis A in rats. There are several possible explanations of the fact that the lesions seem to have escaped the notice of most workers. It might be that their incidence is as variable as, for instance, that of pyelitis. They might be among the later sequelae of the deficiency, developing too late to be detected in the majority of cases before the death of the animal, or they might be one of the early results which go undetected because of the lack of obvious outward manifestations of disease.

It seemed to us that in studying the early lesions of vitamin A deficiency, the central nervous system should be more thoroughly investigated. With this object in view we have carried out during the last two years a series of experiments dealing with the effect of avitaminosis A on the central nervous system of rats, particularly in the early stages of the deficiency. This paper is concerned mainly with the histological results of this investigation, but we have included also clinical observations from two earlier experiments.

PRELIMINARY EXPERIMENTS

Occurrence of clinical symptoms of nerve lesions in avitaminosis A. Even when rats have been allowed to become moribund from deficiency of vitamin A, clinical symptoms of nerve lesions are by no means always detectable. Sutton and his colleagues found that in the negative controls the symptoms were frequently masked by extreme ophthalmia, emaciation, and weakness with subsequent early death. They found the highest incidence in rats which were kept 40–50 days on the vitamin A-free diet, and then given low levels of vitamin A after depletion. Aberle found that in a group of 12 rats on vitamin A-deficient diet, the first appearance of paralysis (stage I) occurred in nine of the rats after about 39 days on experiment, and the advanced stage, in four rats, after about 60 days. In our experience, dealing with rats which received the vitamin A-free diet throughout, we have encountered only a few cases in which clinical symptoms were sufficiently marked to be detected by casual observation. In other cases careful testing of the animal's power of walking on glass plates tilted at various angles revealed a more or less marked tendency to slip, but in many instances even this test gave a negative result. In one experiment involving seventy-four rats which were allowed to become moribund on the deficient diet, only five showed obvious symptoms of lameness, inco-ordination and difficulty in climbing into a wire cage. The glass-slope test showed that nine others were distinctly affected, and twenty-five slightly affected. In the remaining thirty-five animals the diagnosis was either completely negative or extremely doubtful, although in eighteen of these cases the survival periods on the deficient diet ranged from 64 to 78 days. For the five rats which were obviously severely affected, the survival periods were 59–79 days. In another experiment on a group of nine rats, five showed obvious symptoms of nerve lesions after only 35–38 days on the diet, and only two of the group failed to show any definite sign of nerve involvement before they had to be killed after 56 and 57 days.

There is thus considerable variability in the incidence of clinical symptoms of nerve lesions in rats receiving no vitamin A. In many cases even close examination fails to detect any definite symptom, and it is easy to understand how the condition has escaped the notice of many workers. In our earlier experiments no systematic investigation of the central nervous system was carried out, but extensive degeneration was found, by the Marchi method, in the spinal cord of some of the animals which showed clinical symptoms of nerve lesions.

EXPERIMENTAL

The general plan of the work, of which a preliminary account has already been published [Irving & Richards, 1937], was as follows: Albino or hooded Lister rats bred from stock were placed at weaning on a vitamin A-free diet, and killed at definite intervals thereafter, irrespective of their clinical condition. The central nervous system was examined for degeneration by Marchi's method and the results compared with those found in litter-mates, killed at the same time, which had received vitamin A or carotene in addition to the minus A diet.

Histological technique. The rats were killed with coal gas, the brain and spinal cord removed, and definite levels stained by Marchi's method. Many writers have commented on the unreliability of the Marchi technique, and Sutton, Setterfield and Krauss have stated that it is completely unsatisfactory. We have, however, found in this study that, provided certain precautions are taken, very constant results can be obtained. It is scarcely necessary to state that great care must be taken in dissection to avoid any straining or distortion of the tissues which may give rise to artefacts. In our later work, when we concentrated on the medulla, and rejected the other parts of the central nervous system, the following technique was adopted. The skull and upper cervical vertebrae were removed, the cranium and spinal canal opened, and the skull with the central nervous system inside was suspended in dichromate for 12–14 days before completing the dissection. Owing to the tension of the ligaments the cervical vertebrae were pulled at an angle to the skull, the medulla thus having a right-angled bend. To avoid this, the axis of the skull was aligned to that of the spinal canal by splinting it with a match. In using the Marchi technique, formol fixation was avoided, since we have found that this gives very unsatisfactory results. The tissues were fixed in 2·5% potassium bichromate, transferred to osmic acid in bichromate, washed, dehydrated with alcohol, cleared in cedar oil, and embedded in paraffin. Sections were cut at 10μ. Using this technique we have found that in the vitamin A-deficient animals the droplets of reduced osmic acid always occurred in definite tracts, the same tracts being always affected, as is described in more detail later, and not scattered haphazard; that the number of droplets corresponded to the length of time the animals had been on the experimental diet, and that an entirely different picture was obtained with the control animals, which showed very few droplets, no more than were obtained from sections of brains of stock rats. Further, some of our preparations, instead of being treated by the Marchi technique, were sectioned on the freezing microtome after formalin fixation: examination under crossed Nicols, which was kindly carried out for us by Dr J. G. Greenfield, showed that true degenerated fibres were present. We therefore feel confident that the Marchi technique, as used in this way, accurately demonstrates degenerated nerve fibres.

The lesions in the central nervous system. In our preliminary communication we reported findings in the medulla only. But in order to determine what other parts of the central nervous system were involved, semi-serial sections of the entire brain and spinal cord of three rats in advanced stages of vitamin A deficiency were made. On examination these revealed that no central degeneration was to be found rostral to the restiform body save in the optic tract. As a result, histological work was confined to the medulla and cord, and later, for reasons given below, to the medulla alone.

The most striking finding in the medulla was the identity of the nerve lesions in all cases, the only difference being one of degree; and the differences found depended closely on the previous treatment of the animals.

In all cases in which lesions were found, the funiculus praedorsalis was involved. Since very little is known of the anatomy of the rat's brain, it may be well to give in brief the reasons for assigning this name to the tract affected. It is hoped to publish a fuller account of the neurological findings later.

If serial sections of the medulla were studied from just above the sensory decussation to about 1 mm. below the pyramidal decussation, degeneration was found to begin as a few fibres in the region of the posterior longitudinal bundle, and in lower sections to extend over the whole area of the fillet. At the level of the sensory decussation, however, while the sensory fibres were passing dorsally to the nuclei gracilis and cuneatus, the degenerated fibres passed ventrally; at the level of the pyramidal decussation they lay just lateral to the decussation and passing farther ventrally finally occupied the ventro-lateral columns when the pyramidal decussation was complete, as it is in the rat.

The degeneration was usually maximal at the level of, or just below, the pyramidal decussation. At lower levels in the cervical cord the degeneration gradually became less and more diffusely spread throughout the entire white matter.

The only known publication dealing with the central nervous system of the lower mammals is Winkler and Potter's atlas of the rabbit brain [1911]. The brain of this animal closely resembles that of the rat. In this work, a tract is described which follows the course of the degenerated fibres of the avitaminous rat brain. This is known as the funiculus praedorsalis and arises from the decussation of Meynert in the mid-brain. It would thus appear to be a motor tract, corresponding to the tecto-spinal tract in man.

In order to get some further information as to the nature of this tract, the spinal cord was divided in four normal rats at about the 10th thoracic segment. After being allowed to survive for from 5 to 11 days, the medullas were stained by Marchi's method and sectioned. An entirely different picture was obtained. The chief degeneration was in the spino-cerebellar tracts and in the column of Goll, while in the area of the funiculus praedorsalis very few scattered degenerated fibres were seen. We thus found our surmise to be confirmed that the first lesion was in a motor tract; and our supposition in our previous communication that the tracts involved were the spino-thalamic and spino-tectal was not correct.

As the lesions became more advanced, the number of degenerated fibres in the funiculus praedorsalis increased, the degeneration spread farther up the medulla and down the cervical cord, and it was possible to assess semiquantitatively the maximum degree of degeneration found at the pyramidal decussation level and in the upper cervical cord. This has been done throughout in this paper, arbitrary units being employed as shown in Table I.

In more advanced cases the posterior columns and especially the column of Burdach, and the spino-cerebellar tracts became affected, though the degeneration was never as intense as in the funiculus praedorsalis.

The degeneration in the spinal cord was much less uniform than that in the medulla. It differed in its intensity from segment to segment and usually was much less marked than that in the medulla; further, it did not seem to be regularly confined to particular tracts. In view of this lack of uniformity we confined our attention solely to the lesions in the medulla. This variability probably explains the contradictory results obtained by workers who have examined only the spinal cord in this condition.

Experimental data. For convenience in presentation and discussion, the results have been summarized in sections, each section comprising the data for comparable animals. Thus in Section I*a* are given the results for male rats, with little reserve store of vitamin A, placed at weaning on our usual vitamin A-free ration containing wheat starch. Taking these results as our basis of comparison, we have investigated to what extent they are affected by the alteration of various factors in the experimental conditions, e.g. by giving a vitamin A-free diet which contains nothing of cereal origin, instead of the usual wheat starch diet; by giving access to a full diet before weaning; and by using female rats instead of male, as experimental animals.

SECTION I*a*. *Male rats on vitamin A-free diet (wheat starch). Restricted diet before weaning*

The mothers of the experimental animals were stock rats, maintained throughout on stock diet until the 16th day after parturition. From this date, in order to ensure that the young had no large reserve store of vitamin A, the diet placed in the cage was changed to one containing little or no vitamin A. In some cases the diet given consisted of fresh skimmed milk and a mixture of oats, wheat and dried skimmed milk. In other cases the experimental vitamin A-free diet was given, sometimes with and sometimes without fresh skimmed milk. These slight differences in the pre-weaning diet seemed to have no effect on the experimental results. In all cases the mother was removed from the cage 2–3 times daily for half-hour periods, and given stock diet with greens and whole milk. This method we have found very satisfactory in providing young, healthy at weaning but with small reserves of vitamin A, while causing no damage to the mothers. The young were weaned at 23 days, and placed on the vitamin A-free diet, which consisted of caseinogen 18, wheat starch 50, cotton-seed oil 15, salts (McCollum 185) 5, dried yeast 18·3 and lemon juice 5, with a small amount of potassium iodide solution and sufficient water to make into a stiff paste. Vitamin D was supplied as radiostol (0·02 ml. per rat twice weekly).

Positive controls. Some of the positive controls were given daily about 20 μg. carotene in cotton-seed oil. Others were given a vitamin A concentrate in arachis oil, the daily dose being about 20 I.U. vitamin A. A few were given about 50 μg. carotene in arachis oil.

In Section I*a* of Table I are summed up the results for fifty-five male rats which were killed after periods varying from 7 to 51 days on the vitamin A-free diet, and twenty-one positive controls.

The similarity of the results throughout the whole series of animals on the vitamin A-free diet is in marked contrast with the variability in the clinical symptoms of such animals recorded in our earlier work. Many of the animals were killed before there was any loss of weight, or even any definite slackening of the weight curve. In certain of these cases blinking and slight wateriness indicated some degree of abnormality in the eyes, but in others there was no outward sign of the avitaminosis. Nevertheless, from 21 days onwards the animals on the deficient diet showed a progressive degeneration of the funiculus praedorsalis as described above, the degree of degeneration corresponding roughly with the time the animal had been maintained on the deficient diet. At 21 days the degeneration was marked only at the motor decussation level and extended but a short distance into the cervical cord. Of the nine animals killed at this age three showed very slight degeneration (stages 1– or 1); three others showed stage 2, and the remaining three stage 3. At 28–31 days the degeneration began slightly higher in the medulla, was more intense at the decussation level, and extended farther into the cord. The fourteen animals of this age had all reached stages 2 or 3 of the degeneration. From 35 days onwards the degeneration was still more intense, and spread farther up and down the medulla and cord, the posterior columns being now severely affected. At 35–37 days the degeneration reached stages 3 and 4, while animals killed at later dates, viz. 40–51 days, with one exception, showed stage 4 degeneration.

Positive controls. Twenty-one positive controls in this group, which were killed after periods varying from 7 to 51 days, showed only a few scattered black fibres. This condition (stages 1 – or 1) was not progressive. For animals killed at 28 days and later the contrast between positives and negatives was very marked.

SECTION I*b*. *Male rats on vitamin A-free diet* (*tapioca-glucose*). *Restricted diet before weaning*

Cereal toxicity. Considerable attention has been directed to the part played by cereal toxicity in causing nerve degenerative changes. As a result of his earlier experiments Mellanby considered that the onset of these changes was determined by a combination of high cereal intake and absence of vitamin A from the diet. Zimmerman [1933] claimed that such changes can be produced in rats on minus A diet even when no cereal is eaten. It is to be pointed out, however, that Zimmerman's diet contained corn starch, and while it may be the case, as stated by Sutton, Setterfield and Krauss, that in the commercial preparation of corn starch toxic substances, if present, would be removed, it nevertheless seemed advisable to determine whether the nerve degenerations we have described could be produced on a vitamin A-free diet which contained nothing of cereal origin.

An attempt was therefore made to carry out an experiment in which the wheat starch of the basal ration was replaced by potato starch. This experiment had to be abandoned because of the rats' dislike for the potato starch diet. After testing various other root starches, viz. arrowroot, tapioca, sago and tous-les-mois, it was found that the young rats would eat quite readily a ration in which the wheat starch was replaced by a mixture of two-thirds tapioca starch and one-third pure glucose. An experiment was then carried out on eleven male rats which were given from weaning this tapioca-glucose vitamin A-free diet. This ration, with the addition of fresh skimmed milk, was the only diet placed in the cage from the 15th day after parturition. One of the rats was given a dose of 20 μg. carotene daily after weaning. Only two of the animals were killed at 22 days, and these showed the very slight degeneration which was found in two of the rats on wheat starch diet at 21 days. The results for the rats killed at 29 and 36 days were practically indistinguishable from those found on the wheat starch ration, as may be seen from Section I*b*

Table I. Degree of severity of lesions in the funiculus praedorsalis after varying periods on vitamin A-free diet.
The severity of the lesions is indicated by a number according to the following scheme:

Stage 1 – : a very few fibres.
Stage 1: a few scattered fibres.
Stage 2: well marked degeneration.
Stage 3: very marked degeneration.
Stage 4: heavy degeneration.

					Negatives		Positives	
Section	Sex of rats	Experimental diet	Pre-weaning diet	Days on diet	No. of rats	Maximum degree of severity of lesions in the funiculus praedorsalis of individual rats	No. of rats	Maximum degree of severity of lesions
I*a*	♂	Wheat-starch minus A diet	Restricted	7	2	1 –, 1 –	1	1 –
				14	2	1 –, 1	1	1
				21	9	1 –, 1 –, 1, 2, 2, 2, 3, 3, 3	2	1, 1
				28–31	14	2, 2, 2, 2, 2, 2, 2, 3, 3, 3, 3, 3, 3, 3	4	1 –, 1, 1, 1
				35–37	12	3, 3, 3, 3, 3, 3 +, 3 +, 4, 4, 4, 4, 4	8	1 –, 1 –, 1 –, 1 –, 1, 1, 1, 1
				40–43	8	4, 4, 4, 4, 4, 4, 4, 4	4	1 –, 1 –, 1, 1
				46–51	8	3, 4, 4, 4, 4, 4, 4, 4	1	1
I*b*	♂	Tapioca-glucose minus A diet	Restricted	22	2	1 –, 1		
				29	4	1, 2, 3, 4		
				36	4	3, 4, 4, 4	1	1
II*a*	♂	Wheat-starch minus A diet	Full	21–23	3	1, 1, 1 +	2	1 –, 1
				28–30	8	1, 1, 1 +, 1 +, 2, 2, 2 +, 3 (Y)	2	1 –, 1
				35–37	8	1, 1, 2, 3, 3, 3, 4, 4	4	0, 1 –, 1, 1
				42–43	3	3 (G), 3, 4	2	1 –, 1 –
				48–49	3	4, 4, 4		
II*b*	♂	Tapioca-glucose minus A diet	Full	21	2	1, 1 +	1	1
				28–29	2	1 (M), 3 (O)	2	1 –, 1 –
				35	4	1 – (M), 2 (L), 3, 3 + (O)	2	1 –, 1 –
				40–45	3	3, 3 +, 4 (O)	2	1, 1
III*a*	♀	Wheat-starch minus A diet	Restricted	21–22	4	1 –, 1 –, 1 –, 1 –	2	1 –, 1
				28–29	4	2, 2, 3, 3 +	2	1, 1
				35	4	3, 3, 3, 4	1	1
				42	5	3, 4, 4, 4, 4	5	1 –, 1, 1, 1, 1
III*b*	♀	Wheat-starch minus A diet	Full	21	—	—	1	1
				28–29	3	1, 1, 1 +	1	1
				35–36	5	1, 1, 2, 2, 2	2	1 –, 1
				42–45	8	2, 2, 2, 2, 2, 2 +, 3, 4	3	1 –, 1 –, 1
				49	1	4		
III*c*	♀	Tapioca-glucose minus A diet	Full	28–29	3	1 (M), 2, 2 (L)		
				36	2	3 (O), 3 (O)		
				42	1	2 +	1	1 –

of Table I. In the positive control, killed at 36 days, the degeneration, as before, was stage 1. It does not appear, therefore, that cereal toxicity plays any part in the production of the lesions we have described.

SECTION II. *Male rats on vitamin A-free diet. Diet not restricted before weaning*

Effect of pre-weaning diet on time of appearance of lesions. In these experiments there was no restriction in the pre-weaning diet, the young having access either to stock diet (in the case of some of the wheat starch group), or to the basal vitamin A-free diet supplemented by greens and whole milk. The groups consisted of twenty-five rats on the wheat starch ration, and eleven on the tapioca-glucose diet. Results are given also for a number of positive controls.

Comparison of Section I*a* with Section II*a* shows that on the wheat starch basal ration the change from restricted to full diet before weaning has caused a lag of a week or more in the development of the nerve lesions. Thus on the full diet no rat had reached even stage 2 of the degeneration at 21 days, and only one out of eight had reached stage 3 at 28 days, whereas on the restricted diet three out of nine had reached stage 3 at 21 days, and seven out of fourteen at 28 days. Further, on the full diet two rats had not yet got beyond stage 1 at 35 days, another had reached only stage 2 at this age, and two had reached only stage 3 at 42 days. On the restricted diet all rats had reached at least stage 3 at 35–37 days, nearly half of them being stage 4; and at 40–43 days all were stage 4.

It is to be noted that on the full pre-weaning diet the variations between different litters are greater than on the restricted diet. Occasional litters may show little or no lag in the development of the lesions, while in others the lag may be 2–3 weeks. Thus a rat of litter Y on the wheat starch diet showed as marked degeneration at 28 days as one of litter G at 42 days. This variation between litters was even more marked for the few litters on the tapioca-glucose diet (Section II*b*). Thus litter O, of which members were killed at 29, 35 and 45 days, showed lesions as marked as wheat starch rats of the same age which had received the restricted diet before weaning. On the other hand, litter M showed a very pronounced delay in the development of the lesions, and even at 35 days the degeneration present was very slight. Litter L was intermediate in its behaviour between litters O and M. Individual members of one litter were practically alike in their behaviour, although there were such marked variations between the different litters.

This delay in the development of nerve lesions when the young rats have been allowed access to a full diet before weaning corresponds approximately to the increased length of survival period which we have observed in earlier experiments under similar conditions. Thus, in one experiment on seventeen male rats which had been on restricted diet before weaning, the first animal was killed at 40 days. 88 % of the animals were killed by 59 days, and the average survival period for the group was 53·9 days. In another experiment on thirty-eight rats which were on full diet before weaning, the first animal was killed at 49 days; only 47 % of the animals were killed by 59 days, and the average survival period for the group was 61·4 days. For the experiments recorded in this paper it is not possible to give survival percentages, since most of the animals were killed at earlier periods of the deficiency. From a study of the weight curves and the clinical conditions of the animals, however, it was obvious that similar differences in survival period would have been found in these rats also if they had been allowed to become moribund. Of the rats in Section I*a*, which had been on restricted diet before weaning, five were killed in a practically moribund condition before 42 days on the diet. On the other hand, from a study of the weight curves of the rats in Section II*a*, it seems very improbable that any deaths would have occurred in this group by 42 days.

Positive controls. In Section I*a* it was recorded that the positive controls showed a very few scattered black fibres in the medulla. It was thought that this might be due to the fact that, like the minus A rats, the controls got little vitamin A prior to weaning. Even when the diet before weaning was unrestricted, however, this same picture was found in most cases in the positive controls. As explained above, this would appear to be inescapable with the Marchi technique, since we have found the same with stock rats on an adequate diet.

SECTION III. *Female rats on vitamin A-free diet*

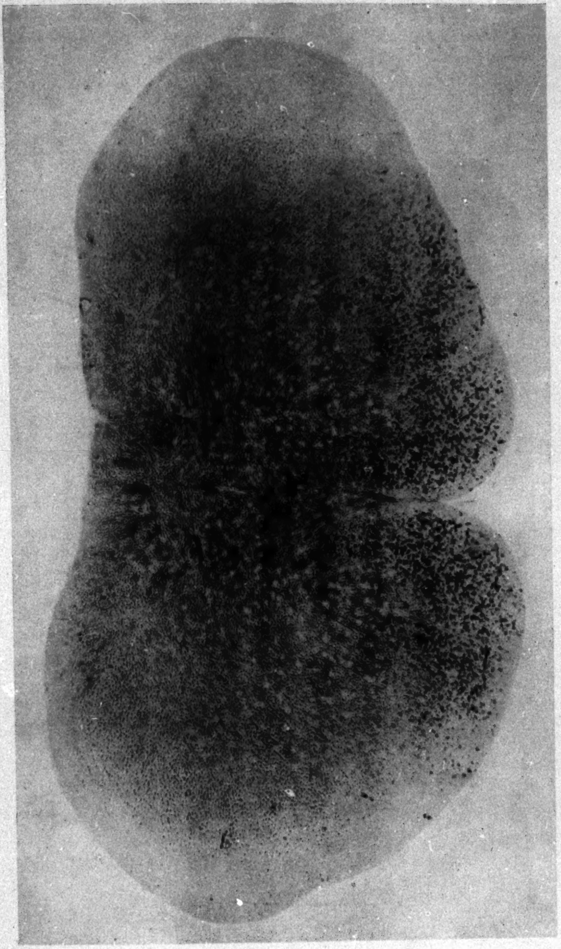
Influence of sex on the time of appearance of the nerve lesions. In Section III of Table I are given the results for thirty-four female rats on the wheat starch minus A diet. Half of these had been on restricted diet before weaning, and half on full diet. A few data are available also for female rats placed on the tapioca-glucose minus A diet after being on full diet, i.e. basal ration + supplements of greens and milk, up to weaning (Section III*c*).

From a comparison of Section I*a* with Section III*a* it would seem that for rats on restricted diet before weaning, the females lag a little behind the males, particularly in the early stages, in the development of the lesions. Thus at 21 days none of the females had got beyond stage 1, while a third of the males reached stage 2, and another third stage 3. From 28 days onwards the difference between the sexes is slight. For rats on full diet before weaning, as may be seen by comparing Sections II*a* and III*b*, the lag of the females behind the males is quite pronounced up to 42 days, and seems to amount roughly to 10–14 days. This lag may probably be explained by the slower rate of growth of the females, and the slower exhaustion of any reserve stores of vitamin A present in the animal at weaning. This slower development of the lesions in the females is also in accordance with previous observations on survival periods of rats on minus A diet. Thus in a group of fifteen females, on restricted diet before weaning, the first animal was killed at 34 days, 60 % were killed by 59 days, and the average survival period for the group was 56·7 days, as compared with 53·9 days for the males. For a group of thirty-three females on unrestricted diet before weaning, the differences were much greater. The first animal was killed at 53 days, only 12 % were killed by 59 days, and the average survival period was 70·9 days.

SECTION IV. *Relation between rate of growth and appearance of lesions*

It is a common assumption that the weight curve of a rat on a vitamin A-free diet is an index of the degree of avitaminosis A in the animal. We have found that the severity of the lesion in the medulla, which appears from our work to be characteristic of this avitaminosis, bears little relation to the weight curve. As we have already indicated, the lesions have been found very frequently in animals which were still increasing in weight, and showing little, if any, outward sign of vitamin A deficiency. In some cases marked lesions have been found even in animals whose weights were equal to, or even greater than, those of the control litter-mates which had received vitamin A from weaning. In Table II are given the weekly weights of a number of typical cases, together with the degree of degeneration which was noted in the medulla. Positive controls have been included when available.

It will be noted that the degree of degeneration may have reached stage 3+ or 4 even in animals whose weight curves are indistinguishable from those of the corresponding positive controls. (see Pl. I). These findings confirm fully the conclusion of Richards & Simpson [1934] that in rats on vitamin A-free diet, pathological conditions may be far advanced before any indication of their existence is seen in the weight curve.



Microphotograph of section of the medulla (just below the pyramidal decussation) of female rat, *h* 42 – (see Table II).

Table II. Weights of rats in sections I–III. The number of the rat indicates its litter, sex (capital letter for male), and the period in days on the experimental diet after weaning. The + sign indicates positive controls, and the – sign rats which received no vitamin A

No. of rat	Section	Degree of degeneration	Weights (in g.) after given no. of days on diet						
			0	7	14	21	28	35	42
Q 21 –	I*a*	3	37	58	78	89	—	—	—
C 36 –	I*b*	4	36	55	71	83	93	106	—
C 36 +	I*b*	1	32	45	57	72	83	101	—
Y 28 –	II*a*	3	47	68	87	106	125	—	—
O 29 –	II*b*	3	39	54	77	88	101	—	—
a 29 –	III*a*	3 +	39	62	83	100	108	—	—
a 29 +	III*a*	1	40	55	76	96	108	—	—
h 42 –	III*a*	4	36	52	72	85	97	102	111
h 42 +	III*a*	1 –	37	54	73	85	97	99	110

CONCLUSIONS

The most important fact emerging from the data presented above is that of 141 rats on a vitamin A-free diet, 108 showed degenerative changes in exactly the same part of the medulla, the thirty-two exceptions being rats killed too soon to show the lesions. The correlation between the degree of the degeneration and the length of time on the diet was often so close that it was possible to state this period after examination under the microscope. This fact, coupled with the non-occurrence of lesions in animals receiving vitamin A or carotene, would seem to make this degeneration one of the specific changes in avitaminosis A. These uniform findings are in marked contrast to the variability reported in pathological condition and the occurrence of epithelial metaplasia in other parts of the body, and suggest that the medullary changes are fundamental in vitamin A deficiency.

Further, we have found in many cases that these lesions are already well marked in animals still growing well and apparently in good health. This finding agrees closely with the conclusion of Richards & Simpson [1934] that the weight curves of rats on a vitamin A-free diet cannot be regarded as a reliable index of vitamin A depletion. It is generally held that about 10 days have to elapse before degenerated fibres will stain by Marchi's method. In this case, the pathological changes must have started even earlier than would be indicated by the histological appearance. We do not yet know if these changes precede those in other parts of the body; but we have removed many organs from the rats used in these experiments and intend to determine the extent of pathological change in them.

The theory of neurotrophic control, however, is tenable only if a sensory tract is involved, and it would appear from our findings that the first tract affected is a motor one, co-ordinating the movements of the eyes and the rest of the body. It may be significant in this connexion that the earliest external change detectable is often concerned with the eye.

The nerve lesions, at any rate in rats, do not seem to be caused by toxins in cereals, since we have repeated our earlier observations using a non-cereal diet and find the same lesion in the medulla.

The fact, just mentioned, that nerve lesions can occur while growth and apparent health are still being maintained, appears to us to have a more general application in medicine. It is now well recognized that subtle degrees of malnutrition, such as sub-scurvy, can exist, and the newer tests for vitamin A deficiency in man, as revealed by night blindness, suggest that a relative lack of vitamin A may well be widespread though undetected by ordinary clinical methods. It is interesting to speculate as to whether these manifestations are accompanied by organic changes in the body, and to what extent these changes are reversible. We hope to give some attention to this latter question in a further paper.

SUMMARY

1. Rats placed on a vitamin A-free diet at weaning early developed degeneration of the funiculus praedorsalis at the level of the pyramidal decussation. The Marchi technique was employed, and in view of the many criticisms that have been made of this method, reasons are adduced for considering the results obtained as reliable.

2. The lesions developed earlier in animals which were denied access to the mother's diet before weaning than in those not so debarred.

3. Female rats developed the lesion later than did males.

4. The same lesions were found in animals on a vitamin A-free diet which contained no cereals.

5. No lesions were found in the medullas of animals which received the vitamin A-free diet with the daily addition of adequate amounts of carotene or vitamin A.

6. Many of the animals showed marked nerve lesions while still increasing in weight.

7. The uniformity of the degeneration in the medulla, when compared with the variability in pathological condition met with in other parts of the body, leads us to believe that this is one of the fundamental lesions of vitamin A deficiency.

The authors are indebted to Sir John Orr, F.R.S., for his continued interest in this work; to Dr J. G. Greenfield and Dr E. A. Carmichael, National Hospital, Queen Square, for much helpful advice; to Mr A. L. Bacharach, Glaxo Ltd., for advice and supplies of vitamin A concentrate; and to Mr A. M. Rennie, F.R.C.S., for performing the chronic cord section operations.

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ADDENDUM

After this paper was written, Møllgaard [1938, *Tierernährung*, **10**, 214] published an account of his findings in pigs with avitaminosis A. He stresses the importance of avoiding formalin fixation in the Marchi technique. His neurological findings in the medulla approximate to those recorded above.

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THE EFFECT OF CALCIUM AND SOME OTHER FACTORS ON THE EXCITABILITY OF THE ANTERIOR RETRACTOR OF THE BYSSUS OF *MYTILUS EDULIS*

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In a previous paper [Singh, 1938*b*] it has been suggested that the phasic contraction in *Mytilus* muscle is produced by the potassium of the muscle, the tonic contraction by the sodium of the muscle or the medium, and that there is probably a third system—the calcium system—which reduces tone and viscosity during a phasic contraction produced by the potassium system.

In this paper an attempt has been made to elucidate this probable action of calcium during stimulation with alternating current (A.C.), and the effects of some factors related to the mechanism of excitation are described.

Methods

The methods for electrical and chemical stimulation were as described previously. In studying increase or decrease of tone or contracture produced by a substance, extension curves were also recorded. When the muscle is suddenly stretched, it has been found without exception that the distance between the base line and the point where the slow extension begins depends upon the degree of contraction, while the rate of extension is mainly determined by the rate of relaxation in a particular substance or the adaptation. Isometric contraction was also recorded, and the results of the two methods were mutually confirmatory. The method of recording extension curves is a sensitive one, and is especially suitable for studying antagonism between various drugs and ions.

Results

Evidence suggesting liberation of calcium during stimulation with A.C. (10*V*.–10 *sec.*). In the absence of calcium, relaxation after most contractions is slow [Singh, 1938*e*]; this, however, only rarely applies to the relaxation after contractions produced by A.C. If the muscle is immersed in 0·564 *M* NaCl, relaxation may not be retarded; but if the muscle is kept in such a solution which is changed frequently, and stimulated therein, the excitability to A.C. gradually declines, and the relaxation becomes correspondingly prolonged, so that the response to A.C. resembles the response to D.C. If calcium is introduced (0·55 *M* NaCl + 0·01 *M* $CaCl_2$), relaxation again becomes rapid. Thus when the muscle is immersed in calcium-free saline, there is diminution in some factor which increases the excitability to A.C. and the rate of relaxation.

There is evidence of increase in such a factor if the muscle is immersed in a calcium-rich solution. If the calcium content of *Mytilus* saline is increased from 0·01 to 0·02 *M* $CaCl_2$, the excitability to A.C. is increased. Reimmersion of the muscle in *Mytilus* saline containing 0·01 *M* $CaCl_2$ after an hour or two does not result in restoration of the original excitability, there being a permanent increase (five experiments in summer muscles insensitive to 0·113 *M* KCl). If the concentration of calcium is greater than 0·02 *M* $CaCl_2$ (about 0·06 *M* $CaCl_2$), the excitability to A.C. diminishes, but if the original concentration is restored (0·01 *M* $CaCl_2$), a permanent increase in the excitability is usually found (six experiments in muscles sensitive to potassium, 0·113 *M* KCl); correspondingly there is a permanent diminution of tone.

The sodium contracture produced by 0·564 *M* NaCl is neutralized by calcium (0·01 *M* $CaCl_2$) or by A.C. (10 V.–10 sec.). If the muscle is deprived of calcium for some time, A.C. may fail to neutralize the sodium contracture, suggesting liberation of calcium by A.C. [Singh, 1938*b*]. The contractures produced by barium, potassium and cyanide are not neutralized by calcium, and A.C. also fails to neutralize them. If the calcium content is doubled (0·02 *M* $CaCl_2$), then partial neutralization is affected (A.C., 10 V.–5 min.).

An interesting feature of the neutralization of the sodium contracture by a short A.C. stimulus (10 V.–10 sec.) is that the sodium contracture develops some time after the cessation of the current, there being a latent period of 10–60 sec. This shows that one of the processes which leads to inhibition of the sodium contracture is not the same as that which leads to the production of tension. If calcium is liberated as a result of the stimulus, it is a secondary effect and not the effect of the stimulus *per se*. It subsides some time after cessation of the stimulus. This probable liberation takes place some time after the A.C. has begun, as the rate of relaxation after a contraction produced by A.C. (10 V.) is slow if the current is continued only for a short time (2–3 sec.).

This latency in the onset of the sodium contracture after a short A.C. stimulus is probably due to the released calcium, as is suggested by the following observation. The secondary A.C. contracture in calcium-free saline [Singh, 1938*b*] is in reality a sodium contracture to which the muscle adapts soon after its development. As already mentioned, in the absence of calcium the excitability to A.C. is decreased; it increases if calcium is reintroduced. After frequent stimulation with A.C. in the absence of calcium, however, the excitability to A.C. (10V.–10 sec.) increases, and the secondary contracture is abolished (Fig. 1).

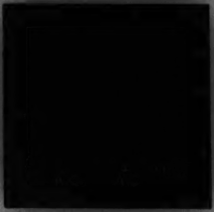

Fig. 1.

Fig. 2.

Fig. 1. Secondary A.C. contracture in calcium-free saline; its abolition with staircase effect. A.C., 10V.–10 sec.

Fig. 2. Secondary A.C. contracture. First three contractions in 0·564 *M* NaCl; the fourth and fifth contractions in 0·544 NaCl + 0·02 $MgCl_2$. A.C., 10V.–10 sec.

Effect of magnesium and potassium on the latent period of secondary A.C. contracture. Magnesium (0·02 *M* $MgCl_2$) diminishes the latent period of the secondary or the sodium contracture, so that it follows soon after cessation of A.C. (Fig. 2). Potassium (0·01–0·02 *M* KCl) increases the latent period. In 0·564 *M* NaCl the latent period is about 5–15 sec. In 0·544 + 0·02 *M* KaCl about 30–60 sec. It is significant to note that potassium increases the latent period of contractions which have been described as due to sodium, viz. the sodium contracture, the secondary A.C. contracture, the contractions produced on stretch and release.

The importance of the latent period of the secondary A.C. contracture lies in the fact that, if the latent period is gradually shortened by increase in the concentration of magnesium, the secondary A.C. or the sodium contracture approaches the A.C. contraction till they fuse, the response to A.C. resembling the response to D.C. The A.C. contraction and the secondary contracture appear to represent the separation of the two components of the D.C. contraction, viz. the contraction produced by the phasic system and that produced by the sodium system.

The secondary A.C. contracture, moreover, demonstrates changes in sensitivity of the muscle to ions present in the fluids bathing it after a short A.C. stimulus. The development of the sodium contracture after such a stimulus shows that the sensitivity of the muscle to ions immediately after A.C. (10 V.–10 sec.) is diminished; thereafter it increases till the sensitivity is greater than previous to stimulation. It then returns to normal.

Consideration of the rate of relaxation of an A.C. contraction [Singh, 1938*e*] during passage of A.C. for 2–3 sec. shows that the sensitivity to ions immediately after the beginning of the A.C. stimulus is high; the diminution in sensitivity is associated with the recovery consequent on the exposure of the muscle to the action of ions during passage of the current. The cycle of changes is diagrammatically represented in Fig. 3. If the stimulating power of ions outside the muscle is great, or if the intensity of exposure to the action of ions during passage of the current is great, as with D.C. or high voltage of A.C., then the recovery is negligible and the relaxation of the contraction is prolonged.

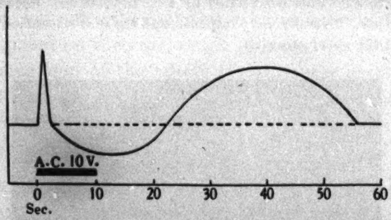

Fig. 3. Diagrammatic representation of sensitivity to sodium ions during and after stimulation with A.C., 10 V.–10 sec. The ordinates are imaginary. The changes in sensitivity during passage of current are judged by the rate of relaxation, and the changes following the passage of the current are judged by the rise and fall of the secondary A.C. contracture. Thick line represents period of stimulation.

Continued stimulation with A.C. Effects are observed analogous to those of the continued action of cations on the excitability to A.C. It is very often found that the tension produced by A.C. rises a second time after it has declined (Fig. 4). In potassium-insensitive muscles the tension does not rise; if the muscle is sensitized by anions that increase the excitability to potassium, such as thio-cyanate and cyanide, the above phenomenon is reproduced. Anions that increase the excitability to potassium diminish adaptation to A.C. This diminution of adaptation shows that as the current is continued the recovery subsides, and the muscle becomes more sensitive to the actions of ions outside. That this secondary rise in tension is due to increased sensitivity to ions outside is shown by the fact that if the current is stopped relaxation is very slow (10–30 min. instead of the usual 10–30 sec., Fig. 4). The above experiments show that the sensitivity to ions during passage of A.C. fluctuates.

Fig. 4 Response to A.C. First contraction by A.C., 10 V.–10 sec. Second contraction by A.C., 10 V.–10 min. Note the wavy response, and the slow relaxation when the current is stopped (at the upper arrow).

Fig. 5. Responses to A.C. (12 V.). Muscle in calcium free saline. Current started at *A*, *C*, and *E*; stopped at *B*, *D*. After *D* the muscle was immersed in *Mytilus* saline containing 0·1 *M* KCl. Note inhibition of inhibition; contracture producing substances diminish adaptation to A.C. Also note the neutralization of the sodium contracture as the A.C. stimulus becomes subliminal.

During continued passage of A.C. not only the tension produced by the current subsides, but also tone and sodium contracture (Fig. 5). The occurrence of the A.C. off-contracture shows that at the termination of the current there is increased sensitivity to ions; during passage of the current some factor counteracts the effect of this increased sensitivity, and since the contracture occurs abruptly on cessation of A.C., the above factor is not the same as that which neutralizes the sodium contracture after cessation of a short A.C. stimulus. The above experiment is interesting in view of the fact that the muscle is simultaneously irresponsive to A.C., to sodium and to the substance responsible for tone. Such an effect is also produced in muscles not very insensitive to potassium (insensitive to 0·113 *M* KCl, but sensitive to 0·16 *M* KCl) by veratrine hydrochloride (1 in 1000, *p*H 7); tone diminishes, the muscle is rendered inexcitable to A.C. and hyperexcitable to potassium. In these muscles veratrine therefore has a differential action on tone, and on excitability to potassium.

Contraction produced on cessation of a short D.C. *stimulus* (14*V*.–10 *sec.*). When the muscle is stimulated with D.C., very often a contraction is produced on cessation of the current. At first it may seem that this contraction should have properties similar to those of the A.C. off-contracture or the potassium contraction. I have not been able to find any evidence that this is so. Substances may diminish or increase the contraction produced during the passage of D.C., the contraction produced on cessation of the current being affected in the opposite or the same direction; but since the contraction on cessation of the current has to be superimposed on the contraction produced by the passage of the current, a comparison of the effects of substances on the former contraction is not possible if the latter contraction does not remain constant. On the contrary, it has been found that the total tension is always affected in the same way as the tension produced by A.C. (Table I), so that

TABLE I. The relation between A.C. and D.C. contractions

		Tension g.			
			D.C. (14V.–10 sec.)		
No. of muscle	In *Mytilus* saline	A.C. (10V.–10 sec.)	During passage	On cessation	Total
1	0·0 *M* KCl	35	30·1	0	30·1
	0·01 *M* KCl	17	11·4	6·8	18·2
	0·02 *M* KCl	7	4·8	10	14·8
	0·03 *M* KCl	0	0	3	3
	0·04 *M* KCl	0	0	1·1	1·1
	0·05 *M* KCl	0	0	0	0
2	0·0 *M* $CaCl_2$	1·2	3·3	4	7·3
	0·01 *M* $CaCl_2$	23·6	12·2	1	13·2
	0·02 *M* $CaCl_2$	34·3	15·1	0	15·1
	0·03 *M* $CaCl_2$	30	12·2	0·5	12·7
	0·04 *M* $CaCl_2$	24·4	11·4	1	11·5
	0·05 *M* $CaCl_2$	17	10·8	1	11·8
	0·07 *M* $CaCl_2$	4·8	6·4	2	8·4
	0·10 *M* $CaCl_2$	1·2	6	2	8
	0·15 *M* $CaCl_2$	1	3	0	3
3	*Mytilus* saline	23	10	1	11
	0·5 *M* NaBr replacing NaCl in *Mytilus* saline	19	5	4	9
	0·5 *M* $NaNO_3$ as above	12	4	2·5	6·5
	0·5 *M* NaI as above	0	0	0	0
4	*Mytilus* saline	15	2·5	0·5	3
	0·1 *M* NaCN	17	6	14	20
5	*Mytilus* saline	26	10	11	21
	1 in 50,000 adrenaline	30	36·5	0	36·5

the contraction on cessation of D.C. is in reality a continuation of the same process as that produced during the passage of the current. Besides the contraction occurs in muscles inexcitable to potassium (0·15 *M* KCl) and in which the A.C. off-contracture is absent.

The occurrence of the above contraction shows that the process which produces tension on passage of the current does not subside with the latter. This is open only to two interpretations: first, as a result of the passage of the current, some substance is liberated (ions such as potassiums, or drugs such as acetylcholine) which takes time to recombine or be destroyed; secondly, the current produces movement of ions, and if an increase in the concentration of ions has taken place in some part of the muscle it takes time for the equilibrium to be restored. The differences between the effect of A.C. and D.C. suggest that excitation is produced as a result of movement of ions; with A.C. there being no after effects, as the ions which move with the current in one direction will move back when the direction of the current is reversed [Winton, 1937].

Contraction produced by distilled water. When the muscle is immersed in distilled water, a powerful contraction giving 30–40 g. tension is sometimes produced. I generally immerse the muscle for a few seconds till the maximum tension has been obtained and the contraction begins to subside; the procedure in no way damages the muscle, the latter being highly irritable to A.C. when reimmersed in *Mytilus* saline. If a contraction is not produced when the muscle is transferred from *Mytilus* saline to distilled water, it can usually be produced by previous immersion in *Mytilus* saline, the osmotic pressure of which has been doubled by adding sodium chloride or sodium bromide. As there is bound to be sudden movement of ions from the muscle into the solution, this contraction may be of importance in connexion with any discussion relating to movement of ions in producing excitation.

Other observations which suggest that excitation is produced as a result of movement of ions are as follows. If a muscle is immersed in a barium-rich solution, there may be no contraction owing to the long latent period. Passage of A.C. or D.C. for 2–3 sec. is sufficient to excite the muscle. This shows that mere presence of barium in the solution is not sufficient to excite the muscle. Similarly sodium is always present in the surrounding solution; it only produces contraction when calcium is excluded.

Alternate changes in excitability to A.C.

Sometimes when the muscle is frequently stimulated with A.C. (10 V.–10 sec. per 1–5 min.) the response alternately waxes and wanes;

for example, in the following experiment the muscle was stimulated with A.C. (10V. – 10 sec. once every 5 min.) and the tension recorded in successive contractions was 10, 12, 15, 10, 7, 11, 16, 10, 15, 11, 7, 10, 15 g.

Similar fluctuations are sometimes seen in continuous tension produced by continued stimulation with A.C. (Fig. 4). The depression in excitability to A.C. leads to a reaction which subsides when the recovery has taken place.

If a potassium-sensitive muscle is stimulated more frequently with A.C. (10V.–10 sec. once per min.), then an interesting phenomenon is seen. After some time the muscle only responds to about one in every five to ten stimuli, so that it was found practically impossible to exhaust the muscle, since it recovered apparently during the phase of inexcitability. Relaxation had become prolonged, so that the tension was maintained during the phase of inexcitability by slow relaxation from the previous contraction. This experiment is in agreement with the tetanus theory of tonus [Bayliss, 1928], and it is further supported by study of the contraction produced by barium.

Effect of calcium on the barium contraction

Alternate changes in the excitability of the muscle are best seen by examination of the effect of calcium on the contraction produced by barium. When the muscle is immersed in calcium-free saline containing barium (0·07 *M* $BaCl_2$) it contracts spontaneously, but the rlaxation of each contraction is not complete before the onset of the next, so that an almost continuous tension is produced. If the calcium content is increased, then the relaxation is more complete and in 0·02 *M* $CaCl_2$ the muscle relaxes almost completely each time (Fig. 6).

Each time the muscle contracts relaxation is at first slow, and then suddenly becomes rapid, the phenomenon being not unlike the neutralization of the sodium contracture by A.C. The phenomenon is less marked or absent if calcium is excluded. It appears that increased sensitivity to barium resulting (1) in contraction and (2) in slow relaxation suddenly brings calcium into action, and the barium contraction is neutralized. The antagonistic process must subside as the cycle is repeated.

An interesting feature of the contraction produced by barium is that the development of tension is quite brisk; it is only the relaxation which is slow. Just before the next contraction begins, the relaxation suddenly becomes rapid. If the decreased rate of relaxation be due to increase in viscosity, then the viscosity suddenly drops before the production of tension; it would then appear that for active contraction the viscosity must be low. The phase of slow relaxation or increased viscosity is a phase of inexcitability to barium, as with A.C. in experiments described above.

Fig. 6*a*. Effect of calcium on the contraction produced by barium (0·07 M $BaCl_2$). In 0·02 M $CaCl_2$; note sudden rapid relaxation (marked by arrows) prior to each contraction.

Fig. 6*b*. The same in 0·01 M $CaCl_2$. (The arrow shows movement of the lever while the drum was not in motion when changing from 0·02 to 0·01 M $CaCl_2$.)

Fig. 6*c*. In calcium-free saline; another muscle. The longer the muscle is kept in calcium-free saline the more continuous the tension, suggesting diffusion away of calcium.

If the muscle with a load of 10 g. is made to contract with barium, the load is maintained at a considerable height for 7–10 hr. There is at first a large initial contraction and the relaxation is very slow. As soon as the muscle has relaxed by 5–10 % of its contraction, it contracts again at intervals of 15–75 min., so that a practically constant height or tension is maintained. After some hours the muscle becomes exhausted and the contractions cease, the muscle now relaxes extremely slowly. The muscle dies while it has only relaxed by 50 % of its length.

The effect of sodium resembles that of barium in many ways. Their tonic contractions are more or less similar; they both increase the excitability to A.C., stretch, and adrenaline. The muscle remains irritable if the sodium in the saline is completely replaced with barium.

Intermittent contractions

It has been shown that by lessening the calcium content, that is, by increasing the sensitivity of the muscle, it is possible to pass from a stage when barium produces intermittent tension to a stage when it produces continuous tension.

Most substances that induce tonic contractions in *Mytilus* muscle also induce intermittent contractions under certain conditions; the muscle responds at intervals though it is exposed to the stimulating agent all the time. The stimulus may be chemical or electrical.

These intermittent contractions represent a transitional stage of excitability from a stage when a substance produces continuous tension to a stage when the substance produces no tension. Thus (1) calcium decreases the excitability of the muscle to sodium; if in *Mytilus* saline or sea water containing 0·01 M $CaCl_2$ intermittent contractions are obtained, they are abolished if the calcium content is raised to 0·02 M $CaCl_2$, and continuous tension is produced if calcium is excluded. In frog sartorius, as with potassium-insensitive *Mytilus* muscle, no tension is produced in the presence of calcium, but intermittent contractions occur in the absence of calcium. (2) The sensitivity of *Mytilus* muscle to some sodium salts varies in the order $NaCl < NaBr < NaSCN < NaCN$. In *Mytilus* muscle, if no contraction is produced in *Mytilus* saline, intermittent contractions sometimes occur if the chloride of the saline is replaced with bromide, and continuous tension if replaced with cyanide. In frog sartorius no tension occurs in frog Ringer, intermittent contractions in NaCl, and contracture in NaSCN [Gelhorn, 1931]. (3) The sensitivity to monovalent cations varies in the order $Li < Na < K$; if intermittent contractions are produced with NaCl they are abolished with LiCl, but continuous tension is produced if part of the sodium is replaced with potassium. (4) The sensitivity to caffeine decreases with pH; caffeine

(1 in 200) produces continuous tension at pH 7·8, intermittent contractions at pH 7, and no tension at pH 6·5–6. (5) Similarly, intermittent contractions are sometimes observed during the production of a tonic contraction. If *Mytilus* saline is replaced with calcium-free saline containing barium (0·07 M $BaCl_2$), the muscle begins to contract intermittently, but relaxation becomes more and more incomplete, and finally a continuous tension is produced. If the muscle is now reimmersed in *Mytilus* saline, it contracts intermittently, the base line gradually falls, the intermittent contractions cease, and the muscle is again in a relaxed state.

Intermittent contractions indicate lack of adaptation, so that they are most often produced in solutions to which the muscle adapts slowly, such as barium; they are very rarely produced in substances to which the muscle adapts rapidly, such as ammonium or potassium. If they are produced in *Mytilus* saline or calcium-free saline they are abolished if the sodium chloride is replaced with glucose or lithium chloride, showing that they are due to the exciting action of sodium. They are probably a result of interaction of two systems, the sodium and the calcium or potassium systems. If the sensitivity of the muscle is high, then the action of the chemical stimulating substance predominates and continuous tension is produced. If the sensitivity is low, the calcium system predominates and no tension results. If the sensitivity is intermediate, then the calcium and the sodium systems predominate alternately, and so intermittent contractions are produced.

In a previous paper [Singh, 1938*a*] attention was drawn to the relationship between high sodium content and high tone. In this connexion it is significant to note that the heart muscle contracts intermittently and its sodium and potassium contents are intermediate between those of isolated striated muscle, which is toneless, and isolated unstriated muscle, which possesses tone.

A.C. intermittent contractions. These are produced during passage of A.C. They represent a transitional phase between stages of rapid and slow adaptation. Thus if adaptation to A.C. is rapid in *Mytilus* saline, then intermittent contractions are produced in calcium-free saline (Fig. 7); they indicate diminished adaptation. If adaptation to A.C. is slow, such as in freshly dissected muscles, then intermittent contractions are produced by immersion of the muscle in a sodium-deficient solution. These intermittent contractions are probably the result of interaction of the potassium and sodium or calcium systems. Intermittent contractions produced by sodium are reduced by a sodium-deficient solution, while

A.C. intermittent contractions may be enhanced by such a procedure. The cilia of *Mytilus edulis* continue to beat in the absence of sodium chloride, so that intermittent excitation resulting in ciliary movement is probably produced by the potassium system, and are akin to the A.C. intermittent contractions.

Fig. 7. A.C. intermittent contractions. First response in sea water for 1·5 min.; second response in calcium-free *Mytilus* saline for 12·5 min.; note the off-contracture. A.C. (10V.) begins at each arrow.

Inhibition of tone

A substance which produces contraction followed by relaxation will obviously produce only relaxation if the muscle is already contracted. A substance which produces contraction followed by rapid relaxation will neutralize the effect of a substance which produces contraction with slow relaxation. Now tone may be regarded as a contraction produced by a substance to which adaptation is very slow (adaptation to sodium chloride is very slow), so that substances to which adaptation is rapid inhibit tone. A.C., for example, and certain chemical stimulants produce a contraction followed by rapid relaxation; these inhibit tone, and the inhibition is in reality a relaxation or adaptation process.

It appears that adaptation producing relaxation may be set up with a concentration of the exciting substance which is too small to produce a contraction. Thus in excitable muscles small concentrations of adrenaline and veratrine hydrochloride, and in inexcitable muscles higher concentrations (adrenaline, 1 in 50,000; veratrine, saturated solution in *Mytilus* saline) produce inhibition and increase the rate of extension. Moreover, a subliminal A.C. stimulus (2V.) only produces relaxation, and if the excitability is diminished by exclusion of calcium or immersion in some of the anions, then higher voltages have the same action (see also Fig. 7).

In previous papers it has been suggested that potassium outside the fibres increases and that inside the fibres decreases tone. These views are also supported by experiments on other smooth muscle [Dale, 1913]. In guinea-pig uterus tonus is inhibited by rise of osmotic pressure and increased by addition of water; the former procedure would raise and the latter procedure would lower the concentration of potassium inside the fibres. In *Mytilus* muscle excitability to potassium is ultimately raised by hypotonic, and lowered in hypertonic solutions; in hypotonic solutions the base line rises if the muscle is frequently stimulated with potassium, signifying increase in tone.

Inhibition of inhibition. Inhibition of tone produced by A.C. is counteracted by immersion of the muscle in a potassium-rich solution (Fig. 5). Inhibition, therefore, is a process related to that which produces tension, as potassium antagonizes this also.

DISCUSSION

Liberation of calcium. Many workers are agreed that calcium is liberated as a result of activity [for discussion see Fenn, 1936]. When the muscle is exposed to the action of barium, calcium is probably liberated resulting in adaptation to barium, the process being similar to the recovery from the effect of cations produced during electrical stimulation [Singh, 1938*e*]. The exciting agent to the liberation of calcium appears to be the cations themselves. This view is supported by the observation that this probable liberation of calcium begins some time after the commencement of A.C. (10V.–10 sec.), and subsides some time after cessation of the stimulus. During recovery from the action of excess of cations there is a stage of hyper-excitability; during recovery from the depressent effect of A.C. there is also a stage of hyper-excitability suggesting that, during the passage of the current, the muscle is exposed to an increased concentration of ions. The probable liberation of calcium during electrical stimulation is thus understandable; it produces adaptation.

In this connexion the work of Schiff [1878] is significant. He discovered that stimulation of the vagus failed to inhibit the frog's heart in the absence of calcium, the heart being perfused with 0·6–0·7 % sodium chloride; but the vagus action was restored if the heart was perfused with 1 % sodium chloride. Excess of sodium chloride would then appear to accelerate the liberation of calcium. According to Busquet & Pachon [1908], addition of calcium chloride in extremely small amount is sufficient, under these conditions, to restore the inhibitory action of the vagus nerve.

It is significant to note that in summer muscles compared to winter muscles (1) excitability to A.C. is high, (2) that to potassium and stretch is low, (3) tonus is less, (4) the depressant effect of cations, anions and drugs is less, (5) relaxation is rapid, (6) fatigue to A.C. is rapid, (7) adaptation to A.C. is rapid, (8) summer muscles swell less when immersed in various solutions. Calcium (0·01–0·02 *M* $CaCl_2$) increases the excitability to A.C., decreases that to potassium and stretch, decreases tone, decreases the depressant effect of cations, anions and drugs, increases the rate of relaxation, hastens fatigue, increases adaptation to A.C. and antagonizes the swelling. Summer muscles, then, appear to contain more calcium than winter muscles.

Small concentrations of potassium (0·01–0·02 KCl) have effects similar to those of calcium, but are inconstant and much less potent. The combined effect of 0·01–0·02 *M* KCl and 0·01–0·02 *M* $CaCl_2$ is greater than that of either of them; especially with the D.C. contraction, either of them alone may not be able to hasten relaxation when together they are effective. It is interesting to note that these concentrations of potassium and calcium which inhibit tone are subliminal for excitation.

SUMMARY AND CONCLUSIONS

1. Immersion of the muscle in calcium-free saline diminishes the excitability to alternating current; immersion in calcium-rich saline for some time and then restoration to *Mytilus* saline increases the excitability to alternating current.

2. Alternating current (10 V.–10 sec.) neutralizes the sodium contracture; there is a definite latent period after cessation of the current before the contracture reappears. Magnesium (0·02 *M* $MgCl_2$) decreases and potassium (0·01 *M* KCl) increases this latent period.

3. The sensitivity to sodium ions is increased for the first 2–3 sec. during passage of alternating current; thereafter it decreases. When the current is stopped, there is a stage of hyper-excitability before return to normal.

4. During continued stimulation with alternating current (10 V.–5 min.), the muscle behaves as if it were exposed to the action of excess of cations. There is at first increased sensitivity to ions, followed by a decrease, and then a further increase. This is also suggested by the fact that on cessation of the current there is a stage of hyper-excitability to alternating current before return to normal; when the muscle is exposed to the action of excess of cations, there is a stage of hyper-excitability to alternating current, before the excitability returns to normal.

5. The properties of the contraction produced on cessation of a short direct current stimulus (14 V.–10 sec.) are the same as those of the contractions produced during passage of direct and alternating currents.

6. When the muscle is stimulated continuously or frequently with alternating current, and continuously with barium, the excitability alternately waxes and wanes.

7. Intermittent contractions, produced by alternating current or certain substances, represent a transitional stage of excitability between a stage when these agents produce continuous tension, and a stage when these agents produce no tension.

8. Tone is inhibited by subliminal chemical or electrical (alternating current) stimuli.

9. Tone in *Mytilus* muscle may be regarded as a continuous contraction produced by fusion of contractions, the relaxation of which is very slow, and probably a passive process; such a contraction is produced by sodium chloride and barium chloride, and can be maintained for hours.

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THE UTILIZATION OF VARIOUS METABOLITES (BLOOD FAT AND LACTATE, CARDIAC AND LUNG GLYCOGEN) IN THE AGLYCAEMIC HEART-LUNG PREPARATION

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In studying the utilization of various metabolites by the heart in the heart-lung preparation, previous experimenters who have wished to obtain low sugar concentrations in the blood have done so either by the use of insulin or by allowing a sufficient lapse of time for the circulating sugar to disappear from the preparation. It appeared to us that it would be advantageous to adopt a method which avoids the use of insulin and also permits immediate observations when the preparation is functioning most efficiently, instead of waiting until such time as the sugar disappears. In such a preparation one would be able to determine readily the lactic acid consumption of the preparation by analysis of reservoir samples of blood without the necessary corrections for the amount of lactic acid added by red cells and by lungs to the circuit during the period of observation. The utilization of other substances by the heart can also be studied in such a heart-lung preparation perfused with sugar-free blood. Further, the rate at which they are used can be determined more accurately as they would not be competing with glucose or lactic acid, nor apparently, as will be discussed below, with any other normal source of cardiac energy present in any appreciable amount in the perfusing fluid. A preliminary report of investigations of this kind has already been published [Waters & Griffiths, 1936].

METHODS

Sugar-free blood. Blood from a big donor dog anaesthetized with ether was prevented from clotting by the addition of a suitable amount of heparin. It was immediately centrifuged, and the plasma pipetted from the cells. The cells were well washed on the centrifuge with Ringer's solution, five or six times, until all the sugar had been removed. The plasma was fermented for 10 min. at 37° with about one-quarter of its weight of well-washed baker's yeast. The yeast was removed by centrifugation and the sugarless plasma recombined with the washed cells. In this manner blood, free from sugar, was readily obtained and when used in the circuit of the heart-lung preparation occasioned no obvious abnormality in the behaviour of the heart or lungs. Preliminary tests should be made to ascertain if this treatment of the blood directly affects the utilization of the substrate under study.

Heart-lung preparation. The usual technique was followed, with only minor modifications. The last blood vessels to be ligatured were the inferior vena cava and, immediately afterwards, the descending aorta. The blood remaining in the heart and lungs was largely removed by running into the heart and lungs, via one arm of a Y tube connected with the cannula inserted into the superior vena cava, about 200 c.c. of Ringer's-cells mixture (150 c.c. Ringer's solution, 50 c.c. sugar-free red cells) warmed to 37°. This mixture was largely removed in turn by perfusing the preparation with some of the sugar-free blood, held in the circuit reservoir. In some experiments the use of Ringer's solution was omitted; a larger volume than otherwise of sugar-free blood was then used. These perfusion fluids were allowed to flow to waste through one arm of a Y tube leading from the innominate artery cannula. Finally, this arm of the tube was clamped, and at the same time the other arm unclamped, thus completing the external circuit of the preparation. From the time of placing the ligature on the aorta to the completion of the circuit of the preparation, procedures were carried out with all possible speed. All animals used had been fasted for 24 hr. and were anaesthetized with ether. Heparin was used as anticoagulant throughout.

Our experiments have been of short duration, usually not more than 2 hr., and terminated while the heart was still beating strongly and before undue oedema had occurred. We have used a small volume of perfusion fluid (e.g. 300 c.c.). In some experiments ventilation was carried out using air containing 5% CO_2, while in others ordinary air was used.

Chemical methods. The methods used for the analyses of blood were, for sugar, Shaffer & Somogyi [1933], for fat, Stoddard & Drury [1929], for lactate, Friedeman, Cotonio & Shaffer [1927]. All glycogen estimations were made by the method of Good, Kramer & Somogyi [1933].

Controls. To provide control tissues, the heart and lungs were removed from anaesthetized bleeder dogs, immediately after the collection of a moderate amount of blood. In a few instances samples of tissues were taken from smaller dogs without initial bleeding, and after performing the preliminary steps in the isolation of the heart-lung preparation. The results with these animals were substantially the same as with the less rigidly controlled animals.

RESULTS

Random samples of lung from blood donor dogs have been taken and their glycogen content determined. A fairly even distribution of glycogen was found, and the concentration in both lungs appears to be about the same. Further, the relative weights of the lungs are of fairly constant value, the average ratio of the weight of the right lung to that of the left lung being 1·40. These results are recorded in Table I.

TABLE I. Ratio of the weights, and glycogen contents, of the lungs of normal dogs

Weights of lungs, g.		Ratio	Glycogen, mg./100 g.	
Right	Left	Right/Left	Right	Left
85·0	60·0	1·42	—	—
58·5	43·0	1·36	—	—
49·7	37·8	1·32	—	—
25·5	16·9	1·51	—	—
—	—	—	165	163
—	—	—	121	125
76·1	56·5	1·35	113	107
53·7	36·4	1·48	147	142
52·0	38·8	1·34	123	136
54·8	38·3	1·42	102	119
64·0	45·2	1·42	124	148
31·3	22·0	1·42	95	101
38·0	28·8	1·32	133 122 138	153 129

This ratio has been used while studying changes in lung glycogen in the heart-lung preparation under different conditions. At the outset of the perfusion the left lung is clamped and removed, samples taken for glycogen estimation and its total weight recorded. At the end of the experiment the right lung is similarly treated. From the ratio of the weights of the two lungs we can obtain a fairly good approximation of the amount of oedema, allowance for which is made in assessing any real change in the glycogen content of the lung remaining in the circuit.

Table II gives the glycogen values (as glucose) of samples (3–4 g.) of lungs removed at the outset of perfusion and those of corresponding lungs of the heart-lung preparations. When the correction is applied for oedema, it is seen that in a few experiments there was no loss of glycogen from the lung of the preparation, even in the complete absence of sugar from the blood. In others there appeared to have been an appreciable decrease in the glycogen of the lung left in circuit. We are inclined to

TABLE II. Changes in concentration of lung glycogen in the aglycaemic heart-lung preparation

Duration of perfusion hr.	Weights of lungs, g.		Ratio Right/Left	Conc. glycogen, mg./100 g.		
	Right	Left		Right	Left	Right corrected for oedema
1¾	33·0	19·4	1·70	68	84	84
1¼	56·5	44·5	1·27	143	110	—
1¾	66·0	38·2	1·72	133	152	163
1¼	38·3	25·3	1·51	129	137	138
2¼	27·4	18·2	1·50	82	129	90
2	37·6	20·6	1·83	59	126	77
1½	30·1	18·9	1·59	104	162	118
2	45·7	27·7	1·65	52	84	61
2	46·5	29·1	1·60	94	151	107
2	62·6	33·4	1·88	64	122	86
¾	42·3	28·9	1·46	124	144	129

believe that the variable results can be in part attributed to uneven oedema.

Lactic acid utilization. Two representative experiments only are recorded in detail (Table III). In the complete absence of glucose appreciable quantities of lactate are removed from the perfusion fluid, the absolute amount removed depending upon a number of conditions, including the concentration of lactate in the blood.

TABLE III. Utilization of lactic acid in the aglycaemic heart-lung preparation

	Time of taking sample after completion of circuit min.	Blood glucose conc. mg./100 c.c.	Blood lactic acid conc. mg./100 c.c.	Lactic acid used mg./g. heart/hr.	
A	17	0	66	>4·2	Output of heart minus coronary flow, 225 c.c./min.
	47	0	40	>2·4	Beats 115–120/min.
	107	0	10	—	B.P. 95 mm.Hg.
					Temp. 37°. 5% CO_2 used
					Wt. heart 44·2 g.
B	15	0	148	>4·3	Output of heart minus coronary flow, 170 c.c./min.
	75	0	98	>2·1	Beats 130–140/min.
	135	0	74	—	B.P. 90 mm.Hg.
					Temp. 37°
					Wt. heart 35·2 g.

It should be mentioned here that in two experiments, although the concentration of blood lactate was high and the general experimental conditions similar to those shown in Table III, the maximum lactate utilization amounted to only 1·5 mg./g. heart/hr. In no experiment have we obtained a lower blood lactate concentration than about 10 mg./100 c.c. blood, i.e. not lower than the normal resting blood-lactate level of the intact animal.

Cardiac glycogen. All cardiac glycogen figures reported in this paper, unless stated to the contrary, were obtained on samples (2–4 g.) taken from the apex of the heart. Duplicate and sometimes triplicate samples were taken for glycogen estimation and almost invariably the figures obtained were in good agreement. In twenty experiments, besides determining the glycogen concentration of the ventricular wall in this way, that of the remainder of the heart has also been estimated. The concentration of glycogen found in the whole heart was usually about 10 % lower than that found in the apical samples. In one heart only was it higher, and rarely was it lower by as much as 15 %. All glycogen figures are recorded in terms of the glucose formed after acid hydrolysis. The figures presented are a little higher than those usually recorded for fasting dog hearts.

Table IV. Cardiac glycogen concentrations of aglycaemic heart-lung preparations compared with controls

Controls												
0·72	0·82	0·65	0·80	1·05	0·71	0·73	0·97	0·83	0·68	0·71	0·72	Av. 0·78 g./100 g.
Moderate work. Aglycaemia and low blood lactate												
0·88	0·67	0·74	0·69	0·62	0·76	0·86	0·80	1·02				Av. 0·78 g./100 g.
(¾)	(1¼)	(1½)	(1)	(2)	(2)	(2¼)	(2½)	(2¼)				
Moderate work. Addition of glucose or lactic acid												
0·80	1·15	0·73	0·92	0·70								Av. 0·86 g./100 g.
(1½)	(2¼)	(2)	(1¾)	(2¼)								
Low work. Addition of glucose or lactic acid												
0·79	0·82	0·74	1·03	0·90	0·82							Av. 0·85 g./100 g.
(2)	(2½)	(2½)	(2½)	(1½)	(3)							

Moderate work: output minus coronary flow 150–180 c.c./min. B.P. 85–95 mm. Hg.
Low work: output minus coronary flow 100 c.c./min. B.P. 50–60 mm. Hg.
Figures in brackets denote duration of perfusion in hours.

It is seen from Table IV that hearts of heart-lung preparations, perfused for periods up to about 2 hr. with blood containing no glucose and only small concentrations of lactate, possessed an average glycogen content the same as that of control hearts obtained from bled dogs. Further it is seen that the values obtained in the hearts of the aglycaemic preparations do not differ substantially from those of hearts of preparations perfused also with sugar-free blood as outlined above, but to which glucose or lactate was added either immediately perfusion commenced or, as was usually the case, at the end of the first hour of perfusion. Thus these latter experiments performed with another purpose in view provide additional control glycogen values. A perusal of the individual results will serve to emphasize that there is no significant difference between the average values of the different sets of figures. We therefore conclude that under the conditions of our experiments over a period of 2 hr. there is no significant decrease in the glycogen content of working hearts perfused with blood which, as will be discussed below, must be regarded as virtually devoid of fuel.

Blood fat. Analyses of reservoir blood samples taken from heart-lung preparations are shown in Table V. The prevailing concentrations of glucose and lactic acid are also included.

Table V. Concentration of fats in the plasma of the heart-lung preparation

Time after completing circuit that sample takes min.	Blood glucose mg./100 c.c.	Blood lactic acid mg./100 c.c.	Plasma fatty acids mg./100 c.c.
17	27	51	170
108	3	9	205
16	12	<25	293
76	0	—	300
15	12	20	303
135	0	—	276
*15	64	32	298
135	11	33	298
10	0	35	376
75	0	14	387
17	0	<25	370
57	0	—	374
117	0	—	381

* In this experiment the blood did not receive any preliminary treatment for removal of sugar.

It is thus seen that in the heart-lung preparation neither the lungs nor the heart use any fat from the circulating blood, whether the blood contains sugar, or is entirely free of sugar and the amount of available lactate very limited. Thus even at such times when the heart seems obliged to draw for energy purposes almost entirely on its own fat reserves there appears to be no replenishment of these stores by the fat from the circulating blood. The apparent slight increases in the concentration of plasma fat, as perfusion continues, we attribute to the relatively greater loss of water from the plasma due to oedema.

DISCUSSION

Lactic acid is removed in considerable amounts from the blood of the heart-lung preparation in the complete absence of glucose. Eggleton & Evans [1930] have shown that the lungs do not use lactic acid, hence the disappearance of lactate would be due solely to the activity of the heart tissue.

Cruickshank & McClure [1936] found no evidence for the utilization of amino acids in the aglycaemic heart-lung preparation, but Burns & Cruickshank [1937] under the same conditions report a utilization of added glycine, with production of some urea. Even so, but little energy would seem to be derived from the oxidation of amino acids. We have found no change in the concentration of plasma fats of the blood of the heart-lung preparation, either in the presence or absence of glucose. Thus in the absence of sugar and with low concentrations of lactate, conditions prevailing in a number of our experiments, the blood provides little known oxidizable substrate, and for practical purposes can be regarded as devoid of fuel for the heart. Under these conditions the heart, and presumably the lungs, derive energy from their own depots.

At present it is impossible to define satisfactorily the metabolic role of the glycogen stores of the heart. That glycogen is being used continuously and as quickly replenished, that it is to be regarded as a potential source of energy in an emergency, or that it is to be treated as part of the general architecture of the cardiac cell, are viewpoints suggested by various investigators.

Visscher & Mulder [1930] found no decrease in the concentration of heart glycogen as a result of hypoglycaemia in their heart-lung preparations, whereas Cruickshank & Startup [1933] found an appreciable decrease (20 % in the first hour) even in slight hypoglycaemia, and Bogue, Evans, Grande & Hsu [1935], using the heart-oxygenator circuit, found the cardiac glycogen to be drawn upon when blood sugar and lactate were low, especially if the expenditure of energy of the heart was high. Also in aglycaemia, without added insulin, Cruickshank & McClure [1936] found a rapid loss of glycogen, amounting to about one-third of that of their control samples.

In our experiments the heart, under conditions of moderate work, for at least 2 hr. appears to lose none of its glycogen stores. This obtains in spite of the sudden deprivation of its supply of sugar and lactate. These findings lend no support to the views of some investigators that cardiac glycogen is being continuously used and as continuously replenished by synthesis from glucose or lactate as in the glycogen of skeletal muscle during activity. We would suggest that only in emergency is the glycogen used; either heavy work or serious deprivation of other sources of energy contained within the heart may involve utilization of glycogen.

Most investigators are agreed that the oxygen consumption of the heart is much greater than can be accounted for by the complete oxidation of all the carbohydrate (glucose and lactate) withdrawn from the blood by the heart. It is probable that fat also is oxidized. Similar considerations seem to apply to the metabolism of the lungs [Shen & Hsieh, 1934]. Cruickshank & McClure [1936] obtained what they considered to be a sufficient decrease in the fat content of the heart of the heart-lung preparation during aglycaemia to warrant the conclusion that the heart was oxidizing fat exclusively. Recently Visscher [1938] after a long series of experiments concludes, with some reservations, that fat is burned by the heart. In a number of our experiments it would seem practically certain that the heart was oxidizing fat almost exclusively. But, surprisingly, none of this fat seems to have been derived from the blood during the course of the experiment. This fact raises the question as to the origin of normal cardiac fat. Blood fat seems the obvious source of supply, so that under normal conditions fat would be continuously withdrawn from the blood by the heart. It is possible that of the total fat of the blood only a fraction and that possibly quite small, can be used directly by cardiac muscle. This small amount of available fat may have been removed before we began our observations, usually about ½ hr. after setting up the preparation. The problem is being further investigated.

Summary

1. A method is described for the speedy removal of sugar from blood, for use in perfusion experiments.

2. Lactate utilization by the heart has been studied in the complete absence of glucose.

3. There is a fairly even distribution of glycogen throughout the two lungs of the dog.

4. In the absence of circulating glucose and with very low concentrations of lactate there is no change in the glycogen content of the heart performing moderate work. Under these conditions there is usually a decrease in the glycogen of the lung.

5. In the presence or absence of available carbohydrate (glucose and lactate) there is no change in the concentration of fats in the blood of the heart-lung preparation.

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THE INFLUENCE OF DIET UPON KETONAEMIA IN PREGNANT EWES

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Introduction

An important cause of mortality in pregnant ewes is variously called "pregnancy disease", "pregnancy toxaemia", or "twin-lamb disease". Its exact aetiology is unknown, although there is general agreement that it is not infectious, that it occurs most frequently in multiple pregnancies, and that it is in some way predisposed to by dietary factors.

The symptomatology is reasonably characteristic. In an affected ewe the gait becomes uncertain and sight is often affected, some animals apparently becoming totally blind. Coma and death usually follow. If parturition occurs the ewe frequently recovers. Post-mortem, the most characteristic feature is pronounced fatty infiltration or degeneration of the liver.

The disease is associated with a marked ketosis, both blood and urine containing excessive amounts of ketone bodies. A strong odour of acetone can often be detected in the breath. Sampson & Hayden [1935] report that in four ewes with pregnancy disease the total ketones in the blood ranged from 36 to 49 mg./100 c.c., whereas in nine normal pregnant ewes the average value was only 3 mg. Allcroft & Green [1938] have found values up to 145·8 mg./100 c.c. in serum from ewes with pregnancy disease.

The ketonaemia is accompanied by a hypoglycaemia. Roderick & Harshfield [1932] found that many ewes with pregnancy disease had blood sugar levels below 30 mg./100 c.c., whereas the value in normal pregnant ewes ranged from 40 to 57 mg. Lyle Stewart [1938] states that in ewes with pregnancy disease, "the blood sugar was frequently, but not invariably, very low".

Among the various possible dietary factors suggested as predisposing to the disease, the classical theory is that of Gilruth [1899], who first described the condition. He held that it is due to overfatness and lack of exercise in pregnant ewes. His views have been supported by McFadyean [1924], Greig [1929], Belschner [1930] and Cameron [1937].

The precisely opposite view, namely, that pregnancy disease is due to under-nutrition of the pregnant ewe, has been supported by Leslie [1931, 1933], Dayus & Weighton [1931], Roderick & Harshfield [1932, 1937], Hopkirk [1934] and McLinden [1937].

A modification of this view was suggested as a result of a survey carried out by the North of Scotland College of Agriculture in its local area. From the replies to a questionnaire issued to farmers who had suffered losses from pregnancy disease, it appeared that protein deficiency in the rations fed might be a predisposing factor.

Finally, field observations made on the Duthie Experimental Stock Farm, attached to the Rowett Institute, suggested that sudden food deprivation, following snow storms, might predispose to the condition.

During the winter of 1937–8, an attempt was made at the Rowett Institute to assess the probable importance of these various suggested dietary factors in the experimental production of ketonaemia in pregnant ewes. Although ketonaemia was chiefly under consideration, observations were made also on the clinical condition of the ewes and on the state of the liver of those dying or killed during the course of the experiment.

The experiments were designed to test the various hypotheses as to the dietary factors predisposing to pregnancy disease which have been outlined above:

(1) The effect of gross over-fatness plus close confinement.

(2) The effect of sudden enforced fast on ewes grossly over-fat and closely confined.

(3) The effect of under-nutrition followed by sudden enforced fast.

(4) The effect of under-nutrition followed by liberal feeding during the last month of pregnancy.

(5) The effect of variation in the protein intake.

METHODS

Grouping. Ninety aged Greyface ewes were used in these experiments. They were kept in pens indoors so that close control of their diet was possible. In October, after being accustomed to the pens, the ewes were mated with a Suffolk ram, and kept under observation throughout their pregnancy.

The ninety ewes were divided into six similar groups of fifteen. The numbering of the groups, their summarized treatment, and the starch and protein equivalents of their rations are shown in Table I.

TABLE I

Group	Summarized treatment	Daily intake Starch equiv. lb.	Daily intake Protein equiv. lb.
1	Fed to over-fatness	1·69	0·24
2	Fed to over-fatness. Periods of fast	1·69	0·24
3	Under-nourished. Period of fast	0·75	0·10
4	Under-nourished.	0·75	0·10
	Period of liberal feeding in last month of pregnancy	1·71	0·40
5	Low protein intake	0·80	0·086
6	Adequate protein intake	0·81	0·154

Blood sampling. In order to study the progressive changes in certain constituents of the blood as pregnancy advanced, the ewes were bled at repeated intervals. The blood was drawn from the jugular vein. Estimations were made soon after the blood was withdrawn. With two exceptions all routine bleedings were carried out between 8.45 and 9.15 a.m.

Estimations made on the blood. Because of the number of animals involved, routine estimations were restricted to those thought likely to be of major importance, namely total ketone bodies and blood sugar. At the commencement of the experiment, midway in pregnancy, and just before lambing, the serum inorganic P, Ca and phosphatase were estimated for each ewe. These were found to be normal for the particular stage in pregnancy.

Analytical methods

Total ketone bodies in blood. The gravimetric method, using Dénigès' reagent, as described by Peters & van Slyke [1931], was employed. From a series of tests made on blood from ketonaemic ewes, it appeared that the "ketone" present was mostly in the form of β-hydroxybutyric acid, as a negligible precipitate was obtained prior to oxidation of the blood filtrate with dichromate solution. For this reason, the figures for "total ketones" in terms of acetone, present in blood, were calculated on the assumption that all the acetone precipitated was obtained from β-hydroxybutyric acid, i.e. the mass of the precipitate in mg. was multiplied by $1\frac{1}{3}$.

Blood sugar. The Somogyi modification of the Shaffer-Hartmann method as described by Peters & van Slyke [1931] was used. Reagent no. 2 was used—this is sensitive to low concentrations of blood sugar.

Serum inorganic P, *serum* Ca, *and serum phosphatase.* The methods adopted at the Rowett Institute [Godden, 1937] were used.

RESULTS

Group I. This group of 15 ewes was fed a ration of 6 lb. turnips, 2 lb. meals and ½ lb. hay per head daily. On this ration the ewes became grossly fat. The average body weight increased from 141 lb. on 27 October 1937 to 191 lb. on 2 March 1938. The blood sugar was consistently high (up to 68 mg./100 c.c.) and ketonaemia was completely absent. Three ewes died from complications due to pregnancy, but none showed any of the symptoms usually associated with pregnancy disease. The livers of the ewes which died appeared macroscopically normal, were shown by chemical analysis to contain the usual lipids in the normal ratios, and on microscopic examination showed no evidence of fatty infiltration. In short, the combination of over-fatness and confinement in these pregnant ewes produced none of the symptoms or pathological changes associated with pregnancy disease of sheep.

Group II. This group of fifteen ewes was treated precisely as Group I except that they were subjected to periods of food deprivation in late pregnancy.

Until the sheep in this group were fasted on 5 February, their history was precisely similar to that of Group I. Their body weight increased, their blood sugar remained at a high level; there was no vestige of ketonaemia. But towards the end of the second day's fast, the ewes became lethargic, and some were too weak to stand. After a 48 hr. fast the ewes were bled prior to refeeding, when it was found that the average figure for blood sugar had fallen by 25% (to 40 mg./100 c.c.) and that two ewes had developed slight ketonaemia. Quarter rations were then supplied but the ewes showed little desire for food, some of them eating practically nothing. On the next day (18 February) transient blindness in some ewes and weakness in all was noted. At 11 a.m. three sheep were in a semi-comatose condition. These animals, however, recovered. By 24 February all the sheep except one, appeared normal. Despite the improvement in appearance it was found that their blood contained a considerable amount of ketone bodies (nine averaged 28 mg./100 c.c.). When the full ration was again supplied the sheep continued to improve rapidly and on 5 March it was found that their blood contained no ketone bodies and that the blood sugar was as high as prior to starvation.

The individual response to fasting was very varied, some sheep within the group showing little change in appearance or in blood constituents, while in others marked abnormalities occurred. Moreover, there appeared to be no close correlation between the clinical appearance of the sheep and ketonaemia or blood sugar level. Thus the ewe (no. 85) which appeared clinically to be most badly affected maintained a normal blood composition, even when semi-comatose; while in another ewe (no. 51) which appeared normal it was found that the blood contained 32 mg./100 c.c. of ketone bodies and that the blood sugar level had fallen from 56 to 37 mg./100 c.c. Ewe no. 85 subsequently lambed with assistance two lambs, which had been dead at least a week. She had to be slaughtered owing to excessive haemorrhage from a torn uterus. Ewe no. 51 lambed normally two good lambs.

A second period of fasting was imposed on 5 March. It was thought probable that the phenomena observed in the first fast would be repeated in an intensified form. The results, however, were different. The clinical symptoms of lethargy, weakness and semi-coma were not observed on this occasion. On the contrary, blood changes, not well marked as a result of the first fast, were much more pronounced. Thus, the average blood sugar level of the entire group fell by 50 %, and four ewes had blood ketones of 13, 13, 15 and 19 mg./100 c.c. respectively. On restoration of the full ration the blood of all the ewes except one returned to normal and all the sheep appeared to be in good health.

Group III. This group of fifteen ewes was fed on a ration of ½ lb. hay mixture, 2½ lb. turnips and 0·8 lb. meals per head per day. On this ration the ewes gained very slightly in weight during pregnancy. Since, under normal conditions, pregnant ewes gain considerably in weight in the latter part of pregnancy (Rowett Institute, unpublished observations), it may be accepted that this group was under-nourished. Moreover, the ewes, towards the end of the experiment were obviously lean and weak. The average body weight at the beginning of the experiment was 142 lb., and at its termination 148 lb.

In contrast to Group I, which was fed to repletion and which showed no ketonaemia, ten out of the fifteen under-nourished ewes in Group III developed ketonaemia of varying degree. This ketonaemia was associated with a hypoglycaemia.

On 25 February a 48 hr. fast was imposed on this group. The first effect was noticed at the 34th hr. of the fast, when one ewe (no. 37) appeared to be blind. By next morning this ewe was unsteady and swayed when made to walk. Two other ewes (nos. 56 and 25) had by this time become blind and weak, one swaying when walking. Other five ewes (nos. 21, 6, 26, 62 and 70) were dull and stood with drooping heads and limp ears. After the fast was ended, the affected ewes recovered, although they took some little time to regain appetite, and failed to regain the weight they had lost.

The period of fasting accentuated the abnormalities of the blood to a marked degree, the ketone bodies increasing by over 100% and the blood sugar dropping to 23 mg./100 c.c.

Subsequent to lambing, it was found that the earlier behaviour of the individual ewes in this group was correlated with the number of lambs they were carrying. Thus, one ewe proved barren. Although she lost 4 lb. in weight during the experimental period, at no time did she show any ketonaemia or hypoglycaemia. Moreover, ketonaemia did not occur except during fasting in the four ewes which bore single lambs. On the other hand, of ten ewes which bore twin lambs, all showed ketonaemia during the course of the experiment, before as well as during fasting. Some of the data for each individual sheep, which are of considerable interest, are shown in Table II.

TABLE II. Total ketones. Data for Group III

No. of ewe	No. of lambs	Total ketones in blood, mg./100 c.c.					
		29/10	26/1	17/2	25/2*	27/2	9/3
91	0	0	0	0	0	0	0
70	1	0	0	0	0	25	19
6	1	0	0	0	0	11	0
54	1	0	0	0	0	11	0
75	1	0	0	0	Tr.	41	0
25	2	0	0	Tr.	13	27	9
48	2	0	0	12	29	Lost	36
17	2	0	0	9	25	48	Tr.
26	2	0	0	9	15	35	Tr.
62	2	0	0	24	24	47	32
19	2	0	0	8	19	39	Tr.
58	2	0	0	14	29	55	20
37	2	0	0	19	25	43	28
21	2	0	0	15	21	43	49
64	2	0	0	35	44	56	Lambed

* Animals were bled at 9 a.m. on the morning of 25 February 1938 and then fasted until 9 a.m. on the morning of 27 February 1938.

Group IV. This group of fifteen ewes received exactly the same ration as Group III until about a month before lambing. During four-fifths of their pregnancy, therefore, they were fed a ration sufficient to maintain weight which, in pregnant ewes, presumes that they were under-nourished. In the last month of pregnancy they were fed a full ration consisting of 6 lb. of turnips, ½ lb. hay mixture and 2 lb. of the meals mixture fed to Group VI, on which they gained rapidly in weight. During the four months these sheep were on a maintenance diet the average gain in body weight was only 5 lb. As pregnancy progressed, ketonaemia and hypoglycaemia became evident in six ewes. All these ewes eventually bore twins. During the first 14 days of being fully fed the average body weight increased by almost a pound a day; the average blood sugar figure increased from 36 to 53 mg./100 c.c. and all traces of blood ketones disappeared. The positive correlation of ketonaemia with twin pregnancy and its negative correlation with dietary improvement is shown in Table III.

TABLE III. Data for Group IV

Index no. of ewe	Change in bodyweight 28. x. 37–17. ii. 37 lb.	No. of lambs	Total ketones in blood, mg./100 c.c.				
			29/10	25/1	16/2*	2/3	10/3
43	+14	0	0	0	0	0	0
15	17	1	0	0	0	0	0
29	13	1	0	0	0	0	0
9	0	1	0	0	0	0	0
39	3	1	0	0	Tr.	0	0
87	30	2	0	0	Tr.	0	Lambed
78	22	2	0	0	9	0	0
30	17	2	0	0	0	0	0
80	12	2	0	0	Tr.	Tr.	Lambed
74	10	2	0	0	44	0	Lambed
47	1	2	0	0	0	0	0
100	2	2	0	0	31	0	0
50	− 4	2	0	0	19	0	0
57	−14	2	0	13	57	Tr.	0
90	−13	2	0	0	45	0	0

* Changed to production ration on 17 February.

Groups V and VI. These two groups, each of fifteen ewes, can be considered conveniently together. The only difference in their experimental treatment was that the protein equivalent of the ration of Group VI was almost double that of Group V, and this difference in experimental treatment made no marked difference to the results. On 19 October 1937 each group showed an average live weight of 139 lb. On 1 March 1938 the average live weights were 150 and 155 lb. for Groups V and VI respectively. The average figures for blood ketones in Group V at successive bleedings were as follows: 0, 0, 17, 23 mg./100 c.c.; in Group VI the corresponding figures were 0, 2, 17, 14 mg. The figures for blood sugar at the last three bleedings were in Group V 39, 40, 35 mg., and in Group VI 39, 39, 40 mg./100 c.c.

Illness occurred in several sheep in both groups. Loss of appetite, a dazed appearance in the mornings and temporary blindness were the main symptoms noted. The sick sheep tended to become worse until they lambed or were slaughtered. Two ewes in Group V (nos. 7 and 35) and one in Group VI (no. 3) were slaughtered when in a dying condition. At post-mortem all these ewes were found to contain two or more lambs and their livers were pale and friable. Chemical and histological examination showed that these livers contained abnormal amounts of fat (58–65 % total lipids on a dry matter basis).

Ketonaemia in relation to changes in body weight of the ewe

From a consideration of the figures both for the averages of groups and for the individuals within groups, it is clear that an inverse correlation exists between ketonaemia on the one hand, and body weight and blood sugar on the other. Thus, with a fall in body weight there is observed a corresponding fall in the blood sugar level, while at the same time the ketones in the blood increase. This relationship is clearly illustrated in Fig. 1, which shows the average body weight, blood sugar, and blood ketones in Group II plotted against time expressed as the duration of the experiment in days.

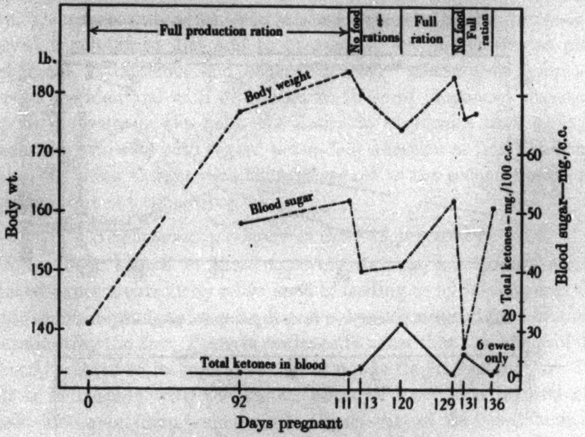

Fig. 1. Effect of starvation on well-fed pregnant ewes.

The same relationship is illustrated in Fig. 2 which is taken from the data of Group III (maintenance with one period of fast) and in Fig. 3 taken from the data of Group IV (maintenance followed by full feed).

It is evident from these figures that ketonaemia tends to increase with under-feeding, still more with fasting, and to decrease when food intake is increased.

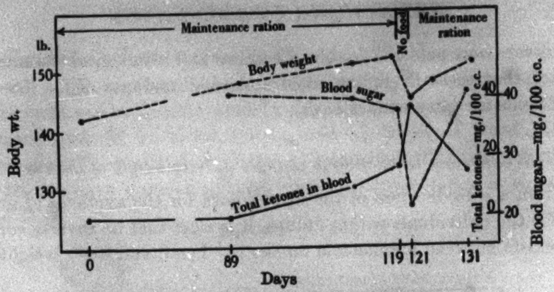

Fig. 2. Effect of starvation on poorly-fed pregnant ewes.

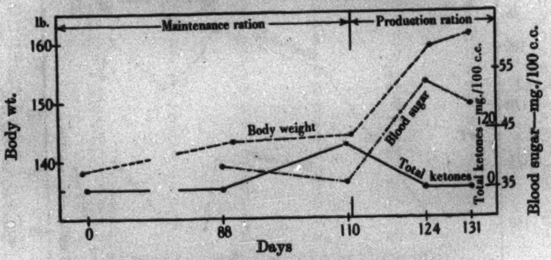

Fig. 3. Effect of increased food on poorly-fed pregnant ewes.

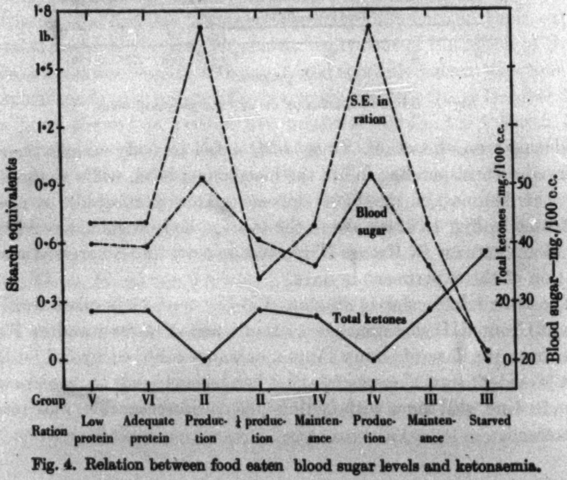

Fig. 4. Relation between food eaten blood sugar levels and ketonaemia.

Ketonaemia in relation to caloric value of the diet

The results of these experiments suggest that there is a definite relationship between the caloric value of the diet and the incidence of ketonaemia (and hypoglycaemia) in the pregnant ewe. This apparent relationship is illustrated in Fig. 4, where it is shown that in all groups the intensity of the ketonaemia varied inversely with the caloric value of the diet and with the level of sugar in the blood.

DISCUSSION

In a general discussion of the results of the experiments described above, it is necessary to consider in the first place whether the ketonaemia observed is related to diet and to pregnancy, and in the second place whether the ketonaemia and other less easily measurable symptoms observed are identical with or related to those of pregnancy disease of sheep as it occurs in the field. We desire to emphasize that while we consider the evidence with regard to the first question as fairly clear and definite, we think that the evidence in regard to the second question is suggestive, but not conclusive.

Ketonaemia in relation to diet and pregnancy

It has been shown in these experiments that ketonaemia can be produced experimentally by a low level of feeding or by fasting, and that a ketonaemia so produced can be dispelled by an increased food intake or by terminating the fast. There is evidence to show that this effect of diet is directly related to its caloric value but not to its protein content. This result is in keeping with the known effect of starvation in producing ketosis. Moreover, in our experiments, the severity of the ketonaemia appeared to be correlated with the loss of body weight in the pregnant ewes.

The results of the experiments also show that ketonaemia is more readily produced in ewes bearing twin lambs than in those bearing singles, and that the ewes which were barren showed no ketonaemia at all. This finding is in keeping with the relationship found between the caloric value of the diet and the incidence of ketonaemia of pregnant sheep since it is known that there is a marked increase in the basal metabolic rate of the mother during the latter part of pregnancy [Harding, 1925], and consequently that the mother's caloric requirements must also increase. Further, Murlin [1910], working with a female bull terrier in successive pregnancies, found that the increase in basal metabolism was proportional to the weight of the offspring at birth. In pregnant ewes, Ritzman & Benedict [1931] have found that a rise in basal metabolism of about 18% per unit of surface area occurs during the final three months of pregnancy.

The results of our experiments justify us in concluding that a caloric deficiency of the diet will produce ketonaemia in pregnant ewes more easily in multiple than in single pregnancies, and that the ketonaemia so produced can be dispelled by increasing the caloric value of the diet.

Pregnancy disease of sheep in relation to diet

In our introduction we discussed the various theories which have been advanced as to the dietary factors causing or predisposing to pregnancy disease of sheep. Our experimental results give support to some but not to others.

They are totally against the commonly held view that pregnancy disease is caused by over-fatness and lack of exercise. In two of our groups, thirty sheep in all, of which the majority bore twins, a degree of over-fatness was produced which is never equalled in commercial sheep. Moreover, the lack of exercise due to confinement in small pens was something much more severe than commercial sheep ever suffer. Yet, except where a period of fasting was deliberately imposed, no ketonaemia or any other symptom suggestive of pregnancy disease was observed.

Nor do our results support the view that protein deficiency is a causative or predisposing factor. Thus there was no apparent difference in the incidence of ketonaemia or other symptoms suggestive of pregnancy disease between the group with a ration of which the protein equivalent was 0·086 and that where it was 0·154. It must be admitted, however, that in the group of fat sheep where ketonaemia was totally absent the protein equivalent of the ration was still higher, namely, 0·24.

Our results do support the view that under-nutrition used in its wider sense, or caloric deficiency in its more restricted meaning, produces ketonaemia in pregnant ewes. They also show that it may produce some of the symptoms commonly held to be characteristic of pregnancy disease, such as temporary blindness and coma. Moreover, in certain ewes showing ketonaemia and which were slaughtered, the livers were found both by chemical and microscopical examination to contain an abnormal amount of fat. The data regarding the livers will be dealt with later in a separate communication. It must be admitted, however, that in individual ewes there was not always a correspondence between the clinical and chemical data. Some ewes with pronounced symptoms suggestive of pregnancy disease showed little or no ketonaemia, while other ewes with severe ketonaemia showed no obvious signs of ill-health. On the other hand, certain field cases of pregnancy disease which we were able to investigate showed a close correspondence in clinical appearance, blood chemistry, and state of the liver to several of the cases of experimental ketonaemia we were able to produce by dietary measures.

SUMMARY

1. With ninety mated ewes, divided into six groups of fifteen, kept in confinement, an attempt was made to produce ketonaemia experimentally, by over-feeding, under-feeding, and by protein deficiency.

2. Pregnant ewes fed to extreme fatness did not develop ketonaemia.

3. A wide variation in the protein content of the diet did not influence the frequency of occurrence or the severity of ketonaemia.

4. The caloric value of the diet was found to be inversely correlated with frequency of occurrence and severity of ketonaemia.

5. Ketonaemia produced by under-nutrition was more frequent and severe in multiple than in single pregnancies.

6. Certain of the symptoms and pathological changes generally considered as characteristic of pregnancy disease were observed in pregnant ewes that were under-nourished or fasted.

7. The relation of experimental ketonaemia produced by under-feeding of pregnant ewes to pregnancy disease of sheep is discussed.

We desire to acknowledge our indebtedness to Messrs A. R. Wannop and A. Macaulay of the North of Scotland College of Agriculture for drawing our attention to field cases. One of us (L. C. S.) is indebted to the University of Western Australia for a Hackett Studentship.

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THE YOUNG CHICK AS TEST FOR THE THYROTROPIC HORMONE

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Ever since the early work of Loeb and of Aron on the thyrotropic hormone of the anterior pituitary lobe, the young immature guinea-pig of about 180 g. body weight has been the accepted test animal for work on this hormone, and its use has continued in spite of the recognition that such guinea-pigs have certain definite disadvantages. Numerous workers, for instance, have called attention to the fact that the more central parts of the thyroid glands of such guinea-pigs frequently show a degree of spontaneous hyperplasia which may readily be mistaken for the effects of a thyrotropically active substance [del Castillo, 1932; McGinty & McCullough, 1936; Cope, 1938*a*].

Because of this a number of investigators have used hypophysectomized rats as more sensitive and reliable test animals for the thyrotropic hormone. The normal rat has naturally a rather hyperplastic thyroid and is relatively insensitive to this hormone. But after removal of the pituitary gland in such rats this thyroid activity subsides and the gland assumes a resting condition with very flat epithelium and intact colloid. Such hypophysectomized rats are good and sensitive test animals for the thyrotropic hormone [Anderson & Collip, 1934; Hertz & Oastler, 1936]. But the operation of hypophysectomy on rats is one which can only be satisfactorily performed after considerable practice, and for this reason such animals have not been available in all laboratories where their use was desirable.

It is because of this fact that we feel that a recent suggestion of Smelser [1937] is of special value and well merits wider recognition and consideration. Smelser has found that young, so-called "day-old chicks" have thyroids which are particularly suitable as tests for the thyrotropic hormone, and advocates their use for work in this field. Such young chicks have for some months been in use in this laboratory, and from our experience with them we feel fully justified in advocating their more extended use. The advantages which they present more than outweigh the disadvantages, and it is the purpose of the present paper to discuss these advantages, our experience in this respect fully confirming the claims of Smelser.

THE ADVANTAGES OF CHICKS

Perhaps the greatest advantage of young chicks in the laboratory is their relative cheapness compared with guinea-pigs. For fully half the year, through the winter and spring months, they are readily obtained from poultry farms. Cockerels are much cheaper than pullets and have been found entirely satisfactory. The cost of such "day-old" light cross cockerels averages about one-tenth or less of the cost of young guinea-pigs. Consequently it is possible by using these to construct a weight response curve for a given preparation of thyrotropic hormone, entailing the use of four or five groups of five chicks each, for an outlay equivalent to the price of about two guinea-pigs. The advantage of such birds in work involving the frequent assay of extracts for thyrotropic activity is thus very considerable. Indeed, where such activity is measured by thyroid weight increase, this cheapness is the main advantage over the guinea-pig.

But when histological criteria rather than thyroid weight are being used, the value of the chick is still greater. Smelser claimed as one of the main advantages of chicks that the thyroid gland was of uniform histological appearance throughout, there being no tendency to develop that area of central hyperplasia so frequently seen in young guinea-pigs, and so difficult to assess when present.

This we are able to confirm. In Pl. I, fig. 1, is shown a typical cross-section of the thyroid gland of a chick 10 days old, which shows well this uniformity of structure throughout the gland. Occasionally a slight degree of hyperplasia involving the whole gland is encountered, but in our experience this occurs considerably less frequently than it does in young guinea-pigs. We have never found glands showing any appreciable difference in histological structure between centre and periphery.

In its response by weight increase to the action of thyrotropic hormone injected subcutaneously, the chick thyroid is of the same order of sensitivity as that of the guinea-pig. But as its body weight is only about one-third or less of that of the usual test guinea-pig, and equivalent dosage is proportional to body weight, it is evident that a considerable economy of test material is achieved by using young chicks compared with the amount required for assay on the same number of guinea-pigs.

Finally, chicks when quite young seem to need considerably less space than do guinea-pigs, an additional advantage in a crowded animal house.

Compared with the above advantages the drawbacks of young chicks are relatively unimportant. It is desirable that during their early days of life they be maintained at an equable warm temperature in a heated cage. But such heating is very easily and simply provided by suspending in each cage a carbon filament electric lamp hanging in an open-top tin. The sides of this tin become hot and must be insulated from the chicks by a covering of thin cardboard. If, now, the sides and top of the cage are covered with felt or sacking, the temperature within is easily controlled by varying the amount of such covering. By such simple means we have had no difficulty in maintaining the cage temperature at the desirable 90° F. for the first week, and lowering this by 5° F. in each succeeding week. The mortality of our chicks from all causes in the first 3 weeks of life has been about 2%. It has not been found desirable to use chicks older than this.

The relatively small size of the chick thyroid is perhaps the greatest deterrent to its more extended use, but we have found the difficulty of dealing with such small thyroids to be considerably less than might be anticipated. The small size of the gland certainly tends to make the removal of the thyroid for weighing and sectioning a more delicate procedure than in the guinea-pig, but relatively little practice is required to dissect it out cleanly and rapidly, and we have not found it necessary to use a dissecting microscope for this purpose.

The anatomical relations of the chick thyroid differ considerably from those of the mammal, and a note on its identification and removal may be of value to those unacquainted with its anatomy.

Dissection of the chick thyroid

The chick, killed with chloroform, is fixed on its back on a board by loops round neck and legs. The downy feathers over the trachea and the upper part of the breast are moistened with water and an incision is made with fine scissors in the mid-line of the neck over the trachea. This incision is continued downwards well on to the sternum, passing to the left side of the crop. Fat and lymphoid tissue (thymus) is dissected away from the base of the neck on the left side above the clavicle, exposing a tough transparent membrane which closes the anterior opening of the thoracic cavity and forms the anterior wall of the clavicular air sac. This membrane is cut through with fine scissors to expose the thyroid gland which will be found lying immediately beneath it close to the carotid artery. It appears as a small pinkish spherical globule which assumes a yellowish translucency when its blood is expelled by slight pressure. This translucency serves to distinguish it from the fragments of lymphoid tissue with which it is often associated, and which are more opaque.

Immediately caudal to the thyroid on each side can usually be seen a second smaller yellowish and discrete globule which is the parathyroid gland.

An attempt to remove the left thyroid gland cleanly from its position on the carotid artery is very liable to lead to much bleeding which obscures the field of dissection, and it is far more satisfactory, therefore, to cut out rapidly a miscellaneous mass of tissue containing the thyroid gland and then to dissect the gland free from the adherent pieces of artery and lymphoid tissue. It is also wise to insert a small plug of cotton wool into the bleeding wound to prevent blood running across to the right side and obscuring the field on that side.

To reach the gland on the right side, the crop is pulled from its loose adhesion to the clavicle, thereby exposing the tough membranous wall of the clavicular air sac. This is cut through and the right thyroid is found immediately beneath it close to the carotid artery. The gland and adjacent tissue is cut out quickly, usually with a piece of artery attached, and is dissected clean at leisure well away from the bleeding wound. Bleeding is usually more severe on the right side than the left, and if this is not done the right thyroid is easily obscured by blood from the damaged vessels.

With a little practice the dissection of the gland free from non-thyroid tissue is considerably easier than might be expected from its small size.

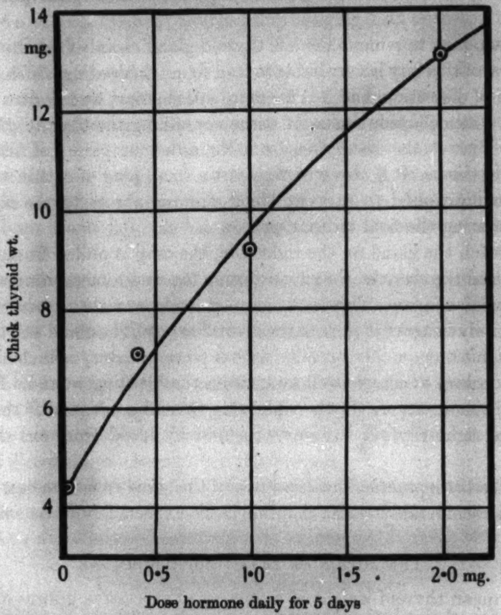
THE ACTION OF THYROTROPIC HORMONE

The mean thyroid weight in a number of successive groups of young chicks is shown in the following table and indicates the degree of constancy which may be obtained:

Date	No. of chicks	Approx. age	Mean thyroid weight (mg.)
Feb. 1938	5	10 days	4·4
Mar. 1938	7	10 days	4·6
May 1938	5	10 days	4·5
Feb. 1938	11	3 weeks	5·4
Apr. 1938	7	3 weeks	4·7

Smelser's chicks at 6 days old gave a mean thyroid weight of only 2·8 mg., which is considerably less than our own. The cause for this difference we have not determined, but it may well be due to difference in the breed of chicks.

The effect of subcutaneously injected thyrotropic hormone on the thyroid of the young chick is essentially similar to that in the guinea-pig. The gland increases considerably in size and vascularity. This weight response provides the most convenient objective method of assay of thyrotropic activity and is considered more fully below. The colloid loses much of its power to stain with eosin, develops a mottled structure, and is finally absorbed completely. The epithelium changes from flat to cubical or definitely columnar with a wide clear protoplasm, and the cell nuclei increase in size, becoming spherical and often showing numerous mitoses. These changes are indicated in Pl. I, figs. 2 and 3. The extent of thyroid weight increase in response to thyrotropic hormone is shown



Text-fig. 1. The weight response curve of the chick thyroid to thyrotropic hormone. (1 mg. is equivalent to 0·3 Parkes unit.)

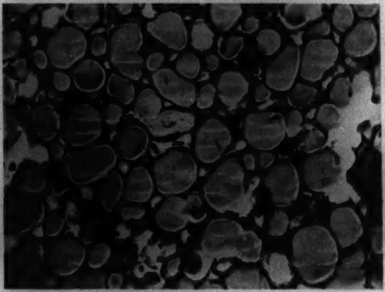


Fig. 1. The normal chick thyroid. × 300.

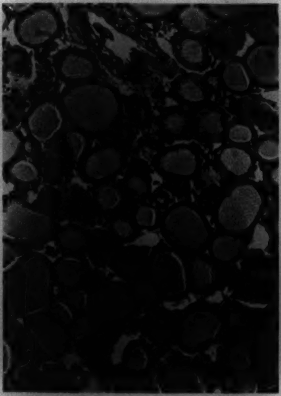


Fig. 2. Chick thyroid response to 0·1 Parkes unit of thyrotropic hormone. × 300.

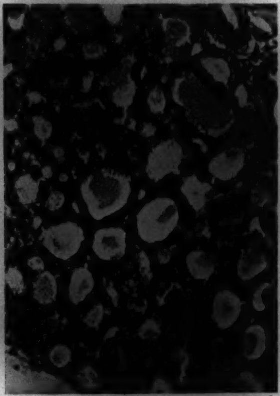


Fig. 3. Response to 0·6 Parkes unit of thyrotropic hormone. × 300.

graphically in Text-fig. 1. In this figure each plotted point represents the mean thyroid weight of a group of five chicks which had been injected subcutaneously with the charted dose of hormone daily for 5 days, and had been killed on the sixth.

In the guinea-pig about 3·3 mg. of this active extract were required daily to double the thyroid weight, whence the preparation contains about 0·3 Parkes unit of hormonal activity per mg. [Rowlands & Parkes, 1934]. From Text-fig. 1 it is seen that 1 mg. or 0·3 Parkes unit will produce a chick thyroid weight of 9·1 mg., that is it will rather more than double the thyroid weight in the chick.

The chicks used in producing this curve averaged 53 g. in weight compared with the 190 g. of the guinea-pig group. It appears, therefore, that the dose of thyrotropic hormone per g. body weight necessary to double the weight of the thyroid gland is approximately the same in the chick as in the guinea-pig, but that by virtue of the difference in body weight only one-third of the quantity is required for the former that is necessary for the latter.

The weight response curve may be used satisfactorily to assay preparations with thyrotropic activity. We have used it recently to assay a preparation obtained from human pituitary glands [Cope, 1938*b*]. This preparation had previously been found by assay on young guinea-pigs to contain 1 Parkes unit in each 12 mg. of powder. 5 mg. of this powder were injected daily for 5 days into each of six chicks. These were killed on the sixth day and gave a mean thyroid weight of 10·8 mg. From the curve this weight corresponds to 0·4 unit, giving the powder a potency of 1 unit per 12·5 mg., a figure which agrees very closely with the result of assay in the guinea-pig.

Chicks can thus be used as easily as, and much more cheaply than, guinea-pigs for the assay of unknown thyrotropic extracts.

That they may also be used for work on antithyrotropic sera is clear from the following experiment which shows that an antithyrotropic serum prepared in the rabbit is as active an inhibitor of thyrotropic activity in the chick as it is in the guinea-pig.

Group	Thyrotropic hormone daily mg.	Rabbit serum daily Normal c.c.	Rabbit serum daily Antithyrotropic c.c.	No. in group	Mean thyroid weight mg.
1	—	—	—	5	4·4
2	—	0·6	—	7	4·6
3	1·5	—	—	9	8·1
4	1·5	0·7	—	12	7·55
5	1·5	—	0·6	5	4·4

From this table it is seen that a daily dose of 0·6 c.c. of an antithyrotropic serum prepared in rabbits was more than adequate completely to inhibit the action of 1·5 mg. or 0·45 Parkes unit daily of a potent thyrotropic extract.

The histological changes in the above series of glands corresponded reasonably well with the changes in weight. The detection of thyrotropic activity by means of the histological changes in the thyroid gland involves a much greater subjective element than does its estimation by the weight increase of the glands. Because of this it is difficult to indicate the minimum dose that can be detected by histological means in the chick thyroid. A daily dose of 0·1 Parkes unit produces a readily detectable rise in mean thyroid weight of a group of five chicks, and this dose will produce well-marked and quite unequivocal hyperplasia in a majority of the glands of the group. But some glands may show relatively little change with this dose, and it is therefore felt that doses of thyrotropic hormone much smaller than this cannot with certainty be detected unless a large group of birds is used.

SUMMARY

Smelser's claim that chicks are convenient test animals for work on the thyrotropic hormone is fully confirmed.

The main advantages of chicks are their much greater cheapness than guinea-pigs, and the greater constancy and simplicity of the histological picture in their thyroid glands during the first 2 weeks of life.

The difficulty of dissecting out cleanly such small thyroid glands is much less than might be expected.

Thyrotropic substances can readily be assayed by the weight increase of the chick thyroid, this weight being approximately doubled by one-third of a Parkes unit of hormone.

The behaviour of antithyrotropic serum in inhibiting the action of the thyrotropic hormone may also be conveniently studied in the young chick.

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THE AIR SPACE OF THE HEN'S EGG AND ITS CHANGES DURING THE PERIOD OF INCUBATION

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The air space of the hen's egg and that of the bird's egg in general, and its changes during the period of incubation, are insufficiently known in many respects, in spite of the fact that its phenomena might be of interest for the study of foetal respiration in the chick. Even its origin is not fully known. According to Lataste [1925] its appearance is the result of contraction of the soft parts from the rigid egg shell as the egg cools after having left the mother's body. Evaporation of water from the surface of the shell was considered to increase its volume gradually, but no importance should in his opinion be attached to this regarding its appearance. It is not easy to understand why evaporation of water should not contribute to its appearance as well as to its expansion, the egg being exposed to both influences simultaneously as soon as it has been laid. Lataste's argumentation that reptile eggs with flexible walls have no air space does not disprove this view.

There are three properties of the air space which interested us especially: (1) the volume and its changes during the period of incubation, (2) the composition of the gas contained and its changes, and (3) pressure of the gas and its changes.

1. Volume of the air space

Some figures relating to the volume of the air space have been given by Hasselbalch [1902]. In order to obtain greater quantities of gas the eggs had previously been kept in a thermostat at a temperature of 25 and 38° C.

The change of volume which the gas space undergoes during incubation has been a subject for investigation as much as a century ago [Dulk, 1830]. Afterwards Preyer [1885] gave some figures. The field was examined more systematically by Aggazzotti [1914], from whose figures Needham in his great work, *Chemical Embryology* [1931], has constructed a curve.

Method. Hasselbalch [1902] measured the volume of the space by measuring the volume of gas aspirated by a small syringe after the shell over the air space had been removed and the membrane perforated by the needle. During this manipulation the egg was kept upright under distilled water, the gas space upwards. Aggazzotti [1914] collected the gas in a funnel after the egg shell had been perforated, the egg being immersed in a saturated solution of sodium chloride made slightly acid with hydrochloric acid. Measurement of the volume was carried out in a calibrated part of the funnel.

Four series of fresh fertile eggs of White Leghorn hens were used in our experiments. The number in each series varied from twenty-five to fifty. Before being put into the incubator they were kept in a cool room for 24 hr. The incubator had a temperature of nearly 40° C.; it did not vary more than ½° in either direction. By a plate covered with moistened cotton a reasonably constant evaporation of water was ensured. The eggs were cooled every day for 5 min. by opening the incubator, the temperature thus falling to room temperature. At the same time they were turned; this turning was repeated about 7 hr. later. It may be remembered that with the chick the time of hatching is 21 days.

The gas was driven out of the air space by mercury and brought into the burette of a 5 c.c. Haldane gas analysis apparatus for measurement. If its volume was smaller than 3 c.c. it was mixed here with a measured volume of nitrogen. Its volume was thus determined with an accuracy of 0·002 c.c. Afterwards it was analysed in the usual way.

In Fig. 1 the apparatus used is reproduced.

The egg, its blunt end upwards, is pressed softly into a layer of glass beads, with which the glass cup C is filled to about one-quarter. A flat iron ring R about 1 cm. broad is placed over the egg and firmly fixed in a stand. Now the cup C and the funnel F are filled with mercury from the levelling tube L by opening stopcock S_1. By using the levelling tube of the gas analysis apparatus the stopcocks S_2 and S_3, the funnel F, the connecting tube t and the burette of the Haldane apparatus are filled with mercury as is shown in the figure. Then the borer B is brought under the mercury surface, and by moving it in a horizontal direction the egg shell is perforated. A limiting disk prevents the point of the borer from penetrating more than 1 mm. into the egg. After the borer has been removed all the gas is collected in the funnel and may be transported to the gas analysis apparatus for measurement and analysis.

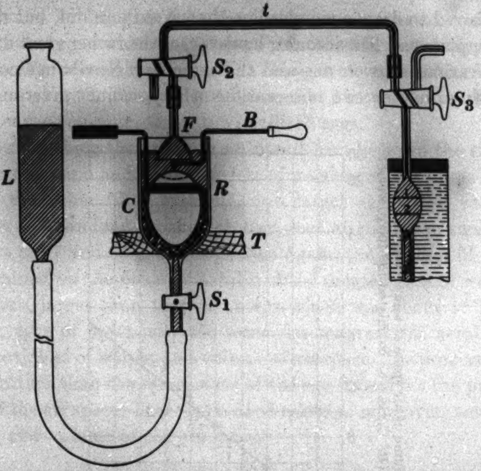

Fig. 1. Apparatus for collecting the gas from the air space, shown just before the egg shell is perforated by the borer (*B*). The gas which will be received by the funnel (*F*) is brought into the Haldane gas analysis apparatus via S_2 and S_3 for measurement and analysis. *L* is the levelling tube filled with mercury. Cup (*C*), funnel (*F*), connecting tube (*t*) and burette are all filled with mercury.

Results. The changes of volume were followed by examining two to eight eggs every day of incubation. The results obtained in the four series agree and will therefore be treated together. Since the eggs varied in weight it seemed desirable to reduce the figures for the gas volume to one and the same weight, viz. 100 g. It was assumed that the pressure in the air space would not differ notably from that of the atmosphere (see under (3)) and accordingly the volumes were reduced to 76 cm. Hg and 40° C. In Fig. 2 a graph is given constructed from the figures obtained in two series.

As Fig. 2 shows, there is a rather wide variation in the volume of air space measured on one and the same day of incubation in various eggs. The volume increases as the period of incubation proceeds, the relation between them being rectilinear. In this respect our curve differs from that constructed by Needham from Aggazzotti's figures, which had an

S-shape, showing the greatest increase of air-space volume between about the 6th and the 13th day. Starting from Aggazzotti's figures we would have preferred to draw a simpler line than Needham did, but this is not of great importance. On account, however, of the rather great divergence found in various eggs on one and the same day Needham's curve, constructed from one or two observations a day, cannot give an accurate picture.

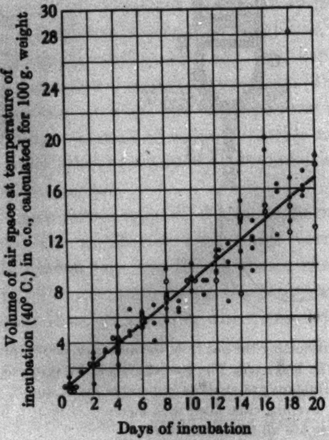

Fig. 2. Increase of the air space during incubation of fertile eggs (•), and unfertilized eggs (o).

A striking difference in slope exists between Aggazzotti's curve and ours. Whereas ours forms an angle of about 40° with the abscissae, the angle of Aggazzotti's curve is less than 30°. Thus the eggs of Aggazzotti's experiments incubated for 18 days had an air space of about 8 c.c., whilst in our experiments the average volume was 11 c.c. (both reduced to 40° C. and 76 cm. Hg). The difference must most probably be attributed to difference in permeability of the shell which varies in animals of different stock. The rather great differences in permeability we found in eggs obtained from one and the same stock, and even from one and the same brood kept under just the same conditions, are doubtless the cause of the wide range within which the volume of air space varies in eggs examined on the same day of incubation.

The question as to how far the increase of volume during hatching is brought about by either the developing foetus or by evaporation of water was examined by bringing into the incubator a series of twenty-eight unimpregnated eggs which for 20 days were treated in exactly the same manner as the impregnated eggs. The changes of volume found here are also given in Fig. 2. The reader will see that the points do not fall far from the curve constructed for the fertilized eggs.

The results show that there is no parallelism between the size of the egg on the one hand and either the loss of weight or the volume of the air space on the other. For example, two larger eggs (72·8 and 75·1 g. respectively), examined on the 4th day, had an air space smaller than two others of 57 and 62 g. respectively. Again, the smallest of the three eggs examined on the 6th day exhibited the greatest loss of weight and an air space, larger than that of an egg which was about 10 % bigger. Moreover, eggs of practically the same size may exhibit notable differences in both loss of weight and volume of air space. The properties of the egg shell rather than the dimensions of the egg determine the progress of volume of the air space. There is no fixed relation, moreover, between loss of weight and volume of the air space.

2. Composition of the gas in the air space

Analyses of the contents of the air space have also been carried out as early as 1830 by Dulk. Preyer [1885] was the first to observe a surprisingly high oxygen content when the eggs had been incubated for some days, no matter whether the eggs were fertile or not. The same observation was made by Hasselbalch [1902]. In incubated fertile eggs he found an average of 21·21 % oxygen, the highest figure being 21·57 %. In infertile eggs about the same maximum was found, whilst the average value corresponded with that of the atmospheric air. The changes of the composition during incubation were investigated by Aggazzotti [1914]. He found a rather high carbon dioxide content (from 1·42 to 2·05 %) in fresh eggs; it decreased during incubation, the oxygen content remaining continually slightly above that of the external air.

We used four series of White Leghorn eggs for our experiments. Since the examination of one egg a day had proved not to give reliable results owing to the wide variation in the figures obtained, forty eggs were taken in every series. Two eggs could thus be examined every day and our conclusions are based on eight eggs examined on the same day of incubation. As the results obtained in the four series corresponded completely, those obtained in one of them (series B) are plotted in the graphs of Fig. 3. The gas analyses were carried out in Haldane's 5 c.c. apparatus. All the foetuses had developed in the normal way and those incubated 18 or 19 days could be reared after having passed another 1–3 days in the incubator.

As Fig. 3 shows, the oxygen percentage falls very slightly (not more than 0·75 %) during the first 10 days of incubation. It then falls rapidly, reaching, by the end of the 2nd week of incubation, an average of 16 % (14 % minimally). During the last week the curve falls farther, the minimum being less than 12 %.

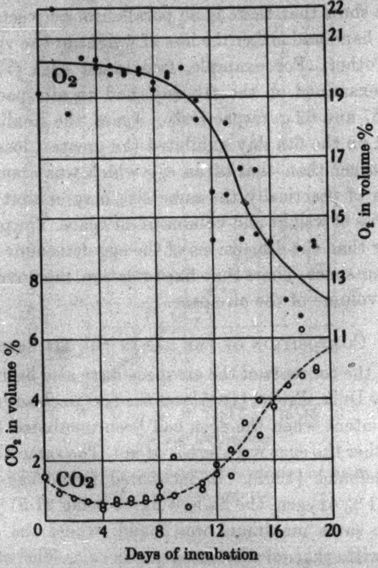

Fig. 3. Composition of the air space of fertile eggs during incubation.

The fall of the carbon dioxide curve during the first few days from about 1·5 to 0·6 % may result from the high carbon dioxide tension in the fresh egg which corresponds to that in the oviduct. The carbon dioxide content is then relatively constant up to the 10th or 11th day. During the following days it increases steeply and reaches a maximum of 6 % at the end.

These results agree rather well with those given by Aggazzotti, except that our figures for both carbon dioxide and oxygen at the end of incubation exceed those given by him. The values are collected in Table I. By using an improved method, which will be described in the following pages, these differences could be shown to be still more prominent than they are here.

TABLE I

Author	Beginning of the experiment		End of the experiment		Days of incubation
	O_2%	CO_2%	O_2%	CO_2%	
Aggazzotti					
Series I after 1 day	20·35	1·89	15·28	4·30	18
„ II after 1¼ days	20·65	1·12	13·65	5·43	17¼
Romijn & Roos					
Series I	22·53	0·51	—	6·69	20
„ II	19·17	1·41	12·72	5·58	20
„ III	22·13	1·76	12·96	5·46	20
„ IV	24·33	0·40	12·17	5·86	20

As our figures in Table I show, the gas in the air space before the eggs have been in the incubator has an oxygen content higher than the atmospheric air. After some hours of incubation it had fallen to 19·5–20%. The difference between the figures at the end of incubation in Aggazzotti's experiments and in ours may be explained, at any rate in part, by the fact that the former are for 18 days of incubation, whereas our measurements were continued to the end of the 20th day.

Moreover, in Aggazzotti's experiments one egg was examined every day of incubation, in each of the two series of eggs used, two eggs being examined occasionally. Now it is clear from Fig. 3 that rather great differences may be met with between two eggs of one series analysed on the same day. For example, two eggs examined on the 14th day exhibited a difference of oxygen content of not less than 2½% (14·24 and 16·63% respectively); the difference between the carbon dioxide figures at the same moment was found to be almost 1% (3·18 and 2·20% respectively). Even at the beginning of the curve a difference is already manifest (see oxygen curve), and after about the 11th day it increases and becomes permanent (see 18th day). In consequence of this fact examination of one egg can no more give a reliable point for the construction of this curve than it did for the volume of the air space. This difficulty can only be partly solved by examining more eggs on the same day of hatching. It must be remembered, also, that in order to collect gas the egg was immersed in salt solution by Aggazzotti, and under mercury by us. During this time ventilation of the egg will be stopped, and, when the metabolism of the foetus has reached an appreciable intensity, say after the 10th day, errors may thereby be introduced. We were not able to reduce the time required to fill the apparatus with mercury and to withdraw the gas from the air chamber to less than 1 min. Table II shows how the gas concentration may be influenced by this procedure. An egg incubated for 16 days or more clearly cannot be kept under mercury or any other liquid for a minute or longer without the composition of the gas in the air space being altered to a noticeable degree.

TABLE II

Number of egg	Days of incubation	Kept under mercury during	O_2%	CO_2%
C 38	16	0 sec.	16·90	2·97
	16	2 min.	16·68	3·24
C 36	19	0 sec.	16·68	4·05
	19	2 min.	16·11	4·35
C 34	20	0 sec.	13·70	4·90
	20	1½ min.	12·96	5·46
	20	5 min.	6·27	7·85

We therefore devised a method whereby the composition of the air space could be followed in one and the same egg during the whole period of incubation. On the blunt end of the egg, disinfected carefully, an apparatus was fixed by which a sample of air could be withdrawn from the air chamber at any moment, the system being closed afterwards until the next experiment. The manipulations did not impair foetal development in any way. The foetuses exhibited normal increase of weight, and after hatching they were reared in the laboratory as normal chicks.

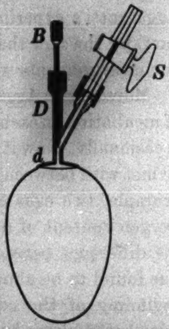

Fig 4. Drilling derrick for collecting gas from the air space. *D*, the derrick; *d*, dish; *B*, borer; *S*, stopcock through which the sample is withdrawn. Magnification about ⅔.

The apparatus is shown in Fig. 4. It consists of a brass dish *d*, 13 mm. in diameter, fixed on the shell. At its centre it supports a narrow brass tube, which we call the "drilling derrick". It contains a borer *B* which emerges from the upper end of the derrick and fits hermetically in a fine rubber stopper covered with a layer of wax. After the egg shell has been perforated and the borer is retracted so as to leave the side tube free, stopcock *S* remaining closed, the layer of wax which covers the stopper is heated a little, thus forming a covering layer in which tube, rubber and borer are involved. Controls showed that closure of the system was complete on both sides, the bottom and the top, at room temperature and also after exposure to the temperature of the incubator. By a vacuum rubber tube a small glass cock *S* was connected with the side tube without leaving any space between. Thus a sample of air could be collected in a sampling tube of 2 c.c. content connected to it. The air sample was analysed in Haldane's 5 c.c. analysis apparatus in the usual way.

The continuous measurement by the drilling derrick method was carried out on six eggs from the 10th day of incubation onwards. Until the 21st day one sample of gas was taken every day. In five of these the chick developed normally until the end. In the 6th the chick was found to be dead on the 20th day of hatching. It had given abnormal figures for the composition of the air space as early as 3 days before, but up to the 16th day it had followed normal development as was also shown by section and by its weight.

The results obtained may be summarized as follows. (*a*) Neither the fall of the oxygen curve nor the rise of the carbon dioxide curve is a continuous one as might be expected. (*b*) In five of the six cases a plateau was exhibited by the oxygen curve as well as by the carbon dioxide curve between the 17th and the 20th day. When, however, the chick perforated the shell before the 20th day the plateau was lacking in the carbon dioxide curve but was observed nevertheless in the oxygen curve. During these days the oxygen and the carbon dioxide content of the air space remained practically unchanged. Subsequently a quick increase of carbon dioxide and a quick fall of oxygen were observed. (*c*) The oxygen curves exhibited a summit at the end of the 12th or the 13th day in four of the six eggs, when the oxygen increased by more than $\frac{1}{2}$% during one day and continued to fall afterwards. It is not yet possible to explain these observations completely, but it seems plausible to conclude that the points mentioned represent remarkable periods in foetal and placental development in the chick.

In Fig. 5 the results obtained in two eggs are plotted. Here the examination could be continued over a part of the 21st day. In the course of that day four and five samples of gas could be taken respectively. In Fig. 6 we give an example in which the carbon dioxide plateau is lacking in a curve obtained from a chick which opened the egg shell at the beginning of the 20th day. The oxygen plateau is present.

Now it is well known that the chick may be heard squeaking softly mostly on the 20th or on the 21st day, although it has not any direct communication with the atmospheric air, the egg shell being completely intact. This was observed in the eggs (*a*) and (*b*). Thus pulmonary respiration had either appeared beside the allantoic respiration or it had fully replaced it. The samples taken from the air space in this period revealed surprisingly high carbon dioxide figures (the lowest being 5·28 % and the highest 9·11 %), even surpassing the oxygen percentage in one case. The course of the curves during the 21st day is visible in Fig. 5, in which the time scale for that day has been given enlarged, thus indicating hours. Examination of the gas was repeated with intervals of about 1 hr., until the experiment was terminated by the chick's piercing the egg shell.

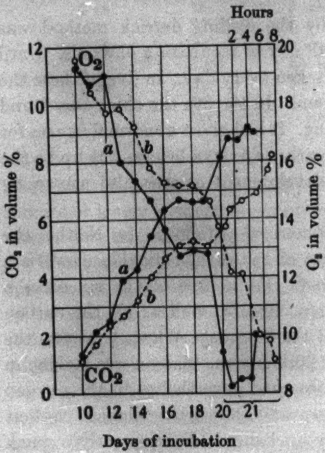

Fig. 5.

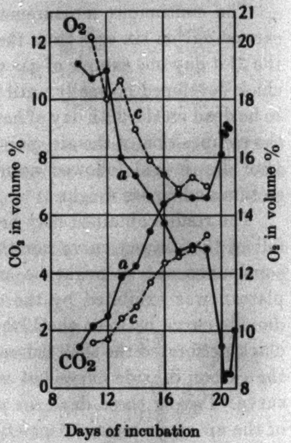

Fig. 6.

Fig 5 Course of the composition of the air space in two eggs (*a*) and (*b*) during the whole period of incubation.

Fig. 6. Course of the composition of the air space in an egg (*c*) in which the shell was perforated by the chick at the beginning of the 20th day; (*a*) is given for comparison.

During the time the chick is squeaking it obviously respires in the air space. No trace of damage to the egg shell could be detected. It is surprising that during this time the animals which had terminated their foetal state respired from an atmosphere which in one case even contained more carbon dioxide than oxygen (9·11 % as against 8·57 %).

Fig. 7 gives a photograph of the air space of an egg which had been incubated 20 days. The chick squeaked. The blunt end of the shell has partly been removed so that the membrane which forms the bottom of the space is visible. Amidst the blood vessels of the allantois which are smaller than they had been before, a tear of the membrane is seen, in which is the beak of the young animal.

Thus it was possible to analyse the atmosphere from which the animal inspired under physiological conditions during the period which apparently lay between the foetal state and the postfoetal, which begins after the egg shell has been left. This interposed period, which may be called the parafoetal period, sets in when the allantoic circulation begins to reduce its respiratory function, and the air space, separated from the atmospheric air by the egg shell, forms a second atmosphere from which the animal respires directly by its lungs. The permeability of the egg shell, as is well known, increases during incubation. This parafoetal period is terminated when the chick perforates the obtuse end of the egg shell with its beak.

Fig. 7. Egg incubated for 20 days. The chick was squeaking. The blunt end of the shell has been partly removed and the rupture of the membrane which separates the gas space from the rest is visible, together with the beak of the young animal.

The composition of the atmosphere in which the chick respires in the parafoetal period is surprising, the oxygen pressure having fallen below half the atmospheric oxygen pressure, and the carbon dioxide pressure surpassing that in atmospheric air 200 times and even more. It must therefore be assumed that a condition of anoxaemia and increase of carbon dioxide content is produced during this time.

3. PRESSURE IN THE AIR SPACE

In order to be able to use the figures given in the preceding pages for the problem of respiration in the avian foetus, it is necessary to know the partial pressure of the gases in the gas chamber, and for this it is necessary to know the total pressure. Aggazzotti, in calculating these partial pressures assumed that the total pressure was that of the atmosphere.

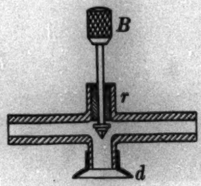

Fig. 8.

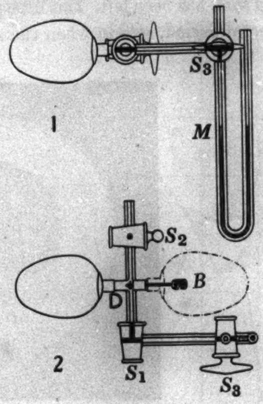

Fig. 9.

Fig. 8. Drilling derrick. *d*, dish; *B*, borer; *r*, rubber stopper covered by a layer of wax. About original size.

Fig. 9. Air space connected to the manometer (*M*) by the drilling derrick (*D*). Pressure can be measured continually. *B*, borer by which the shell is perforated after S_2 is closed. The egg can be turned 180° round the axis S_1–S_2. (1) Seen from the front; (2) seen from above.

The pressure of the gases in the air space has never been determined so far as we know. We used the following apparatus. A drilling derrick, of somewhat different construction than the one described before, was fixed on the blunt end of the egg (Fig. 8). It was connected (see Fig. 9) with a manometer *M* filled with coloured water by capillary glass tube and by a stopcock S_1 which at the same time was the axis round which the egg, together with the drilling derrick, could be rotated 180° without being taken out of the incubator. Thus the egg was turned twice a day without any trouble. The apparatus was fixed on the egg by the brass dish *d*; the borer *B* was made of steel.

After the apparatus had been placed on the egg, stopcock S_2 being opened, the whole was placed in the incubator, and after the temperature had risen to 40°, stopcock S_2 was closed. The shell was then perforated, the borer withdrawn and, by opening S_3, the air space was connected with the manometer. Stopcock S_1 has a T-shaped bore, so that the manometer communicates with the air space in both positions of the egg as shown by Fig. 9 (2). The egg rested on a small table of wire netting which was turned together with the egg.

The apparatus was placed on the egg on the 8th or the 10th day of incubation. The volume of the system by which the volume of the air space was thus increased was 1·526 c.c.

Results. In all ten eggs examined the pressure in the air space was shown to remain relatively constant during the whole time of incubation. It was found to be a little higher than the atmospheric pressure, but the difference was small; it varied from ½ to 3 mm. of water. The figures found for three eggs are given below as representative (Table III).

TABLE III. Pressure in air space (above atmospheric pressure) in mm. water

Days of incubation	Egg 1	Egg 2	Egg 3	Egg C
8	—	—	1½	—
9	—	—	2	—
10	2½	—	—	—
11	2	2	1½	—
12	2	2	1½	—
13	2	1½	1½	—
14	2	—	1½	—
15	1	1½	1½	—
16	2	—	1½	—
17	1	1	1	—
18	2	½	1	2
19	—	—	1½	3
20	—	1½	—	2
21	—	—	—	0 to 2

In the last column figures are given for an egg (C) in which the chick was heard squeaking at the beginning of the 21st day. The water in the manometer moved rather rhythmically to and fro with a frequency of 120 per min., obviously as the result of respiratory movements made by the chick in the air chamber. In another experiment similar rhythmic fluctuations of 2 mm. had a frequency of 100 per min. They could be followed for many hours and came to an end when the chick pierced the shell.

DISCUSSION

When the allantoic vessels develop and cover the shell membrane which forms the base of the air space—they reach full development at the 14th or the 15th day of incubation—the air space becomes important for foetal respiration, its importance increasing with its volume. At the 14th day of incubation it has a volume of 6–7 c.c., which during the last week of incubation increases to 9–11 c.c. The foetus finds here a volume of gas available for allantoic ventilation and separated from its blood by a thin membrane and the capillary walls only. Thereby gas exchange will be relatively intensive between foetus and this atmosphere, from which it is separated by a surface which takes 10–20 % of the respiratory surface of the whole egg shell.

Considering the composition of the gas found in the air space, it may be supposed to be of special importance during the parafoetal period. After the chick tears the shell membrane on the 20th day it respires directly from an atmosphere which contains no more than 8½–15 % of oxygen, carbon dioxide having risen to 5–9 %. Two circumstances are present at this moment by which oxygenation of the foetal blood is deteriorated: (*a*) reduction of the allantoic blood vessels, and (*b*) lung respiration in the atmosphere mentioned.

The composition of the air space during the parafoetal period, and the preceding days, will bring about a state of partial anoxaemia and increased carbon dioxide concentration in the blood. From the observations of Windle & Barcroft [1938] on the effect of various amounts of carbon dioxide and oxygen on the movements of chicks between 13 and 20 days old, we may expect in consequence the following effects: (*a*) increased muscle tonus; (*b*) inhibition of movements which are not a part of the respiratory pattern; (*c*) intensifying of respiratory movements which, within a short time, will have to replace completely ventilation by the allantoic vessels; (*d*) movements of the neck and the trunk by which the egg shell is perforated on the 21st day. Furthermore, Windle & Barcroft observed increased irritability and quickening of all somatic movements as the initial result of carbon dioxide administration or anoxaemia.

These authors state that a respiratory rhythm was never seen before the 17th or 18th day (sometimes not before the 19th), although movements of all the muscles involved in respiration appeared very much earlier. Our observations show that at the 18th day the amount of carbon dioxide in the air space had risen to 5–7 %, and during 2 days, the 18th and 19th of incubation, this concentration was maintained (see Figs. 5 and 6). The plateau formed by the oxygen curve at the same time corresponded with an oxygen content varying from 13 to 15 %. It seems, therefore, that during this constancy in composition of the air space foetal respiration is initiated. The composition, moreover, is such as to induce the respiratory rhythm and the movements by which the air-space membrane is perforated. The air space, therefore, plays an important part in foetal development during that period.

The parafoetal period which follows lasts 24 hr. or more; it is the period in which respiratory rhythm and general muscular activity are further developed. The animal is being prepared for postfoetal life.

SUMMARY

1. The volume of the air space of the hen's egg increases in a rectilinear relation to the time of incubation. At the end of the period of incubation a volume of 11 c.c. may be reached, whilst in the fresh fertile egg it does not exceed some tenths of a c.c.

2. A method has been described by which the composition of the gas found in the air space could be determined in one and the same egg throughout the time of incubation. This procedure is necessary owing to the wide variability between different eggs examined under the same conditions of incubation.

3. The foetus remains under fully physiological conditions during the experiments.

4. A method has been given by which the pressure in the air space was measured in one and the same egg continuously during the time of incubation, development of the foetus remaining normal.

5. When the chick leaves the egg it has terminated its foetal state about 24 hr. previously. During these hours, the parafoetal period, respiration in the air space by pulmonary breathing has been added to allantoic respiration. An exceedingly high carbon dioxide content is present in the air space (up to 9·11 %). On the other hand, oxygen percentage has fallen to 9 and less.

6. The importance of the air space for foetal and parafoetal respiration and for development in general is discussed.

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THE EXPERIMENTAL PRODUCTION OF DEAFNESS IN YOUNG ANIMALS BY DIET

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In a series of publications since 1926 it has been shown that degenerative changes in many nerves of both central and peripheral systems of young animals can be readily produced by dietetic means [Mellanby, 1926, 1930, 1931, 1933, 1934*b*, 1935, 1937, 1938]. The most common lesions consist of demyelination changes followed by complete disappearance of the fibres in many of the afferent peripheral nerves and ascending fibres in the central nervous system. It has been shown that nerve cells are also affected by these dietetic conditions and reasons have been given which suggest that these nerve cell changes may be primary to the demyelination of the nerve fibres. wrong

In the usual experimental period of about four months, the afferent fibres degenerate before the efferent, which may not be much affected, if at all. With a longer dietary period the efferent fibres are affected but never as badly as the afferent.

Although the most obvious lesions consist of changes in the myelin sheath there are also changes in the axis cylinders in the nerves of both dogs and rabbits fed on diets deficient in carotene and vitamin A. These changes can be seen in fibres which show annular myelin degeneration and even in cases where there is no other obvious defect in the nerve. The axis cylinder changes have been studied in more detail in branches of the sensory nerves supplying the dental tissues by King, Lewinsky & Stewart [1938] in vitamin A-deficient rats.

The dietetic conditions favouring these developments are (1) deficiency of vitamin A and carotene, and (2) presence of much cereal. Of these two factors, the absence of vitamin A and carotene is the more important, for in young rats at least, the same demyelination changes can be produced by synthetic diets in the absence of cereal if they are deficient in vitamin A and carotene [Zimmerman, 1933]. Again, the cereal content of the diet may be very high and yet, in the presence of sufficient vitamin A and carotene, little or no nerve degeneration will be produced. On the other hand, changes in the type of cereal eaten or the addition of certain cereal products may alter the degree of degeneration. The experimental diets are made up of ordinary foodstuffs, except for the irradiated ergosterol, and are rich in all substances of the vitamin B complexes.

One of the afferent nerves affected by demyelination changes under these dietetic conditions is the 8th, both cochlear and vestibular divisions, the former more so than the latter. Degenerate changes in the vestibular nerves are associated with inco-ordinate movements of locomotion of the animal, as might be expected. Young dogs showing this condition are also inattentive and do not respond to the call of people at close range, a characteristic which distinguishes them from normal puppies. This inattention is doubtless due partly to deafness.

The present paper is concerned with these abnormal changes due to vitamin A deficiency, and contains a description of the pathological condition of the labyrinthine capsule, both of its nerve supply and the bone itself. When a rich source of vitamin A is added to the diet while every other condition of diet and environment is kept constant, the animals remain normal in behaviour and the labyrinthine capsule escapes pathological change.

All young animals examined up to date, i.e. dogs, rabbits and rats, react to the above dietetic defects, so far as nerve degeneration is concerned, in the same way, differing of course in degree. The new facts, especially concerning bone hyperplasia, described in this paper, refer for the time being to the dog. There is, however, no doubt that bone hyperplasia takes place in the rabbit and probably in the rat, and will be described in a future paper.

HISTOLOGICAL TECHNIQUE

Since the degenerative changes of a nutritional nature in the 8th nerve were first seen and described, the need of following up and examining in detail the internal ear itself has been constantly in mind. Many of the animals fed over a fairly long period on diets very deficient in vitamin A were obviously deaf, and unsuccessful attempts were made to grade the degree of deafness in dogs and rabbits by observing their reactions to sound. The earlier histological preparations made for the detailed examination of the finer structure of the cochlea and vestibule were unsatisfactory. Last year I discussed the subject of deafness in animals on deficient diets with Mr Cleminson, Head of the Ferens Institute for the Study of Otology, and he was good enough to arrange for a demonstration of the technical methods for histological examination of the internal ear in use at that Institute. Mr Peet kindly demonstrated these methods of fixation by perfusion through the heart and gave full details of the further technique. Moreover, serial sections of celloidin embedded material were made from two labyrinths fixed by Mr Peet. These and other sections were carefully examined by Dr Hallpike, to whom I am most grateful for assistance and advice. The Ferens technique has in the main been used in the present work, with the exception that, in order to shorten the time needed for obtaining the final result, paraffin has replaced celloidin embedding in the end process. In many cases formol-saline was used as a fixative agent instead of Wittmaack's solution which contains potassium bichromate. For decalcification 5 % nitric acid was often used instead of 1 %. By these devices the time occupied in preparation was reduced from 4 to 5 months to between 5 and 7 weeks. The resulting histological preparations are not as good as those obtained by the Ferens Institute method, especially as regards nerve cells and epithelial structure, but the great saving of time counterbalances this drawback. As I understand that Dr Hallpike will publish a detailed account of the Ferens Institute method for the histological study of the labyrinth capsule, no further reference will be made to the methods used in the present work.

A total of fifty-one labyrinths from 44 dogs have been examined. Serial sections of sixteen of these from twelve animals have been cut, stained and examined; in some cases when both labyrinths of one animal were examined, pathological changes were not identical. The whole cochlea and vestibule of the remaining cases were cut but not mounted serially. In addition, ground undecalcified sections were prepared from some of the ears for purposes of bone examination. Some experiments of a similar nature have been made also on rabbits and rats and serial sections of the labyrinthine capsules cut. These experiments will not be described in the present publication. It is now evident that because of the complicated structure of this organ and the multiple changes that occur in the labyrinth under the experimental conditions that it would have been easier if serial sections had been made from all the experimental animals, in order to appreciate more fully the actual course of events. This, however, was not possible, but the number of labyrinths serially sectioned is probably sufficient to allow of a general description of the changes produced.

EXPERIMENTAL METHODS AND RESULTS

The experiments on young dogs described in the present paper were not made primarily to test the effects of different diets on the ear. The action of certain forms of diet in producing and preventing extensive nerve degeneration had been well established and most of the experimental feeding was at the time centred on an attempt to get information on the part played by cereals and cereal products in influencing these changes. This explains why, so far as the cereal portion of the diet is concerned, there are small differences in the various series of experiments. It is not the intention in the present publication to deal with this point, however, since the effect of changing the cereal portion of the diet is only one of degree and not of kind. For the time being attention will be mostly confined to the changes in the neurones and bones of the cochlea and labyrinth produced by variations in the vitamin A content of diets rich in cereals. Reference will, however, be made to some animals in whose diet potato wholly replaced the cereal.

The experiments were made under the following conditions.

Litters of puppies varying from 7 to 10 weeks old were given diets consisting of separated milk, cereal (oatmeal, white flour etc.), lean meat, yeast, peanut oil, irradiated ergosterol (vitamin D), orange or lemon juice, and sodium chloride. In this diet there is very little vitamin A or carotene, but it is, as far as is known, otherwise complete.

The time necessary for producing the pathological change varies with the stores of vitamin A in the liver of the puppy at the beginning of the experiment and also with the severity of the dietetic deficiency. Usually, however, the animal's behaviour is abnormal after 2–4 months of such feeding and the changes may be great after 4 months. In the experiments reported in this publication the duration of the dietetic period varied from 4 to 10 months in different animals. The pathological changes were very intense in the dogs which had received vitamin A-deficient diets for the longer periods.

Apart from the specific effects produced by the vitamin A deficiency, the animal's health was usually fairly good, the food was eaten well and the weight steadily increased. Dogs do not readily die of infection in these conditions, as they might be expected to do when one thinks of the multiple infective lesions developed by young rats fed on vitamin A-deficient diets. Nor do dogs develop xerophthalmia as easily as do rats but, when they do, it is a more serious matter, owing to the rapidity with which perforation of the cornea often takes place. The relation of xerophthalmia to degeneration of the trigeminal nerve has been previously described [Mellanby, 1934*a*].

A typical experiment in which advanced degenerative changes were produced will be given first. Three puppies aged 8 weeks were given the following dietary mixture daily over a period of 42 weeks: separated milk powder 12·5 up to 30 g., oatmeal 75 up to 270 g., peanut oil 7·5 up to 15 c.c., meat deprived of visible fat 25 up to 60 g., baker's yeast 3·75 up to 12 g., sodium chloride 1 up to 4 g., vitamin D as irradiated ergosterol 1000 I.U. The former quantity represents the amount of each constituent given at the beginning of the experiment and the latter the amount given when a maximum diet was being eaten. In addition, dog I had daily 30,000 I.U. of vitamin A. Dog II did not get any additional source of vitamin A throughout the feeding period. Dog III received no added vitamin A for the first 15 weeks, but for the last 27 weeks it had 30,000 I.U. daily.

At the end of the experiment the vitamin A content, measured as Carr-Price blue values using a Lovibund tintometer, of the livers of the three dogs was as follows:

Dog	Carr-Price blue values per g. liver	Approx. equivalent in I.U. per g. liver
I (vitamin A-rich diet)	93	2800
II (vitamin A-deficient diet)	Nil	Nil
III (curative experiment)	50	1500

Dog III will not be further discussed here, but will be referred to later in dealing with possible recovery.

Fig. 1 represents a section of the cochlea and its components of dog I (vitamin A-rich diet). Fig. 2 is a corresponding photomicrograph of dog II (no vitamin A). (Only a few of the photomicrographs illustrative of the results obtained were made from dogs I and II. Other illustrations were sometimes chosen, not because they were different, but because they were technically better.)

The most obvious pathological changes found in the labyrinths of young dogs brought up on these vitamin A-deficient diets are (*a*) nerve degeneration, more especially of the cochlear neurones, (*b*) new bony growth in the modiolus, (*c*) overgrowth of the internal periosteal layer of the capsule, i.e. the bone in proximity to the brain, (*d*) serous labyrinthitis, (*e*) degenerative changes of the organ of Corti and sensory epithelium of the semicircular canals. Occasionally a small amount of bony overgrowth was seen in the basal whorl of the scala tympani.

Changes in the 8th nerve (cochlear and vestibular divisions). The cochlear division (or its remains) of the 8th nerve is seen in Figs. 1 and 2.

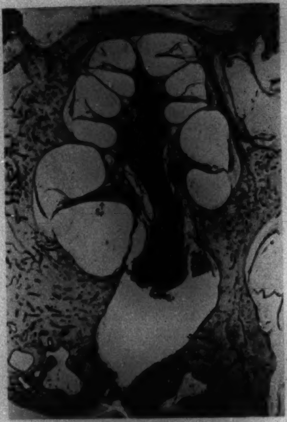

Fig. 1.

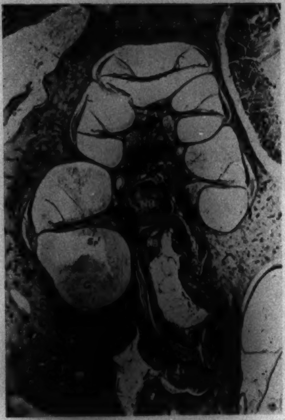

Fig. 2.

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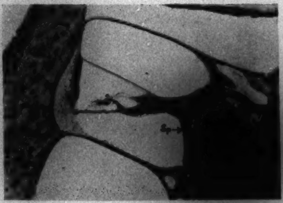

Fig. 3.

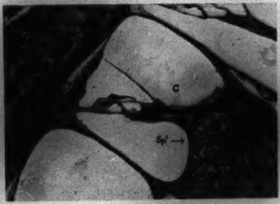

Fig. 4.

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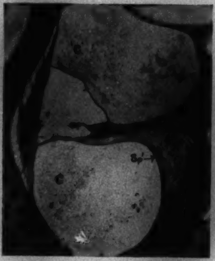


Fig. 5.

Fig. 6.

Fig. 7.

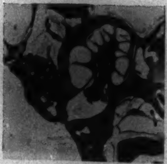


Fig. 8

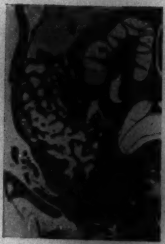


Fig. 9*a*.

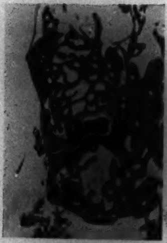


Fig. 9*b*.

In Fig. 2, representing the cochlea of dog II (vitamin A-deficient diet for 10 months), this nerve has disappeared, whereas in Fig. 1 from dog I (vitamin A-rich diet) it is intact. Not only has the cochlear nerve of dog II completely degenerated but the spiral ganglion cells and the nerve fibres to the organ of Corti have gone, and their place is filled with loose connective tissue. The spiral ganglion cells and nerve fibres to the organ of Corti of dog I are normal.

Photomicrographs (×50) of a whorl of a cochlea of each of three dogs are seen in Figs. 3–5. Fig. 3 is normal and is in great contrast to both Figs. 4 and 5, which are from dogs fed on diets deficient in vitamin A. In Fig. 4 the vitamin A-deficient animal was killed after 5 months of the diet. Many cells of the spiral ganglion have disappeared and those remaining are shrunken and the cytoplasm is free from granules. Most of the peripheral branches to the organ of Corti have also gone. In Fig. 5, taken from dog II, which had been on the vitamin A-deficient diet 10 months, the condition is still worse; there are no cells and no fibres left. Figs. 6 and 7 are high-power photomicrographs (×500) showing the detailed condition of the cells of the spiral ganglion in a normal (receiving vitamin A) and a vitamin A-deficient animal respectively. The normal spiral ganglion cells seen in Fig. 6 are very different from those in Fig. 7, which represent part of the spiral ganglion seen in Fig. 4. Comparison between Figs. 3–5 also shows that in the vitamin A-deficient animals (Figs. 4, 5) the peripheral fibres of the cochlear nerve supplying the organ of Corti have degenerated, whereas in the animal receiving vitamin A (Fig. 3) they are present.

It will thus be seen that in an advanced case (Fig. 5) the whole cochlear nerve, including the spiral ganglion cells and their central and peripheral branches, disappears.

In experiments where the nerve degeneration is not as advanced as in the instance just described, the spiral ganglion cells at the base of the helix are usually rather more abnormal than those at the apex; one exception to this rule, where the basal spiral ganglion cells are less affected than the apical cells, was encountered.

Although all the cells of the spiral ganglion of dog II (Fig. 2) were degenerated, there were still some cells in Scarpa's ganglion. It is true that they were in most cases degenerate but they had not completely disappeared as had the cells of the corresponding spiral ganglion. Throughout this investigation it has been found that the cochlear division of the 8th nerve is more affected than the vestibular. The ganglion cells of Scarpa's ganglion in dog I (vitamin A-rich diet) are normal. These results are in keeping with those previously described [Mellanby, 1935], where it was shown that, comparing the number of degenerating fibres in the cochlear and vestibular divisions of the 8th nerve in A-deficient animals, it was always found that the cochlear division suffered more severely than the vestibular division.

Overgrowth of bone of the labyrinthine capsule. A glance at Figs. 1 and 2 shows another striking development in the internal auditory meatus of the vitamin A-deficient dog (Fig. 2). It will be seen that two massive pieces of bone fill up the meatus and leave little or no space for the branch of the 8th nerve. The bone appears on the whole to be of normal structure but the lower piece has a large cavity full of fatty tissue. The corresponding meatus of Fig. 1 (vitamin A-rich diet) is patent and the 8th nerve here has an uninterrupted passage to the brain.

The filling of the internal auditory meatus at the modiolus end seen in Fig. 2 is only found in advanced cases of degeneration produced by the prolonged dietetic deficiency described, but all experimental animals, so far examined, brought up for a few months on similar diets show some such bony change.

Another position where bony overgrowth is found is in the periosteal bone adjacent to the brain and to the internal auditory meatus. This new bone can be seen by comparing Figs. 8, 9*a* and 9*b*, which are low power photographs of the labyrinthine capsule of dog I (Fig. 8) and dog II (Figs. 9*a* and 9*b*). In Fig. 8 (diet rich in vitamin A) the meatus and the cochlear division of the 8th nerve can be traced from the helix of the cochlea to the edge of the capsule. In Fig. 9*a* the meatus is tortuous and the exit of the nerve from the brain cannot be seen, so that several sections are needed to trace its full course. This displacement is illustrated in Figs. 9*a* and 9*b*; Fig. 9*b*, where the exit of the 8th nerve or its remains is clearly seen, represents a section about 2·5 mm. from that shown in Fig. 9*a*.

The depth of bone between the basal whorl of the cochlear helix and the brain is much greater in Fig. 9*a* (from A-deficient animal) than in the control (Fig. 8). The cancellous nature of the new bone is also clearly seen in Figs. 9*a* and 9*b*. This increase in periosteal bone is more clearly demonstrated in Figs. 10 and 11 representing sections cut in a plane at right angles to those of Figs. 8 and 9*a* and 9*b*. It will be seen that whereas in the normal dog (Fig. 10) the bone on each side of the internal auditory meatus is thin, in Fig. 11 it is greatly thickened, due to the laying down of new periosteal bone, which appears to be of the ordinary cancellous type. There is no excess of osteoid tissue, nor is there any evidence of the

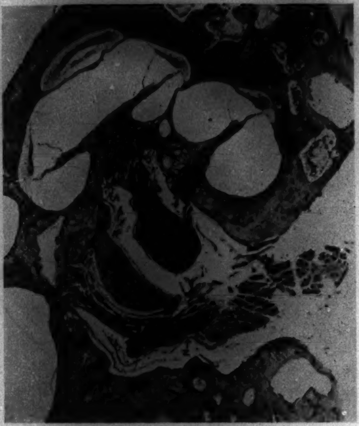
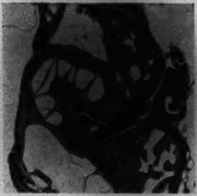


Fig. 12*b*.



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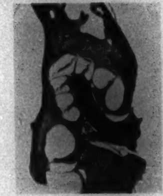

Fig. 10.

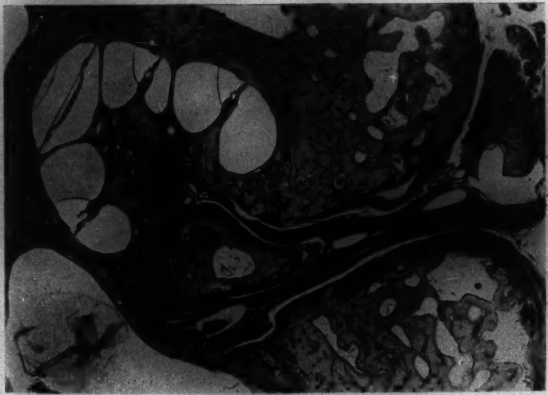

Fig. 12*a*.

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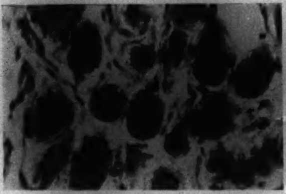

Fig. 13.

Fig. 14.

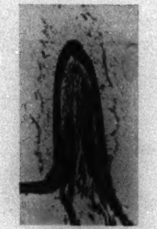

Fig. 15*a*.

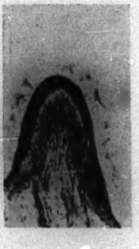

Fig. 16*a*.

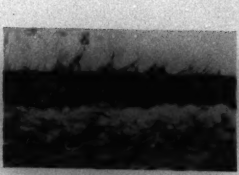

Fig. 15*b*.

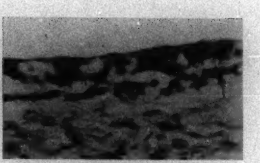

Fig. 16*b*.

changes being inflammatory in origin. The spaces in the bone are abundant in the outer periosteal layer and are usually filled with fat.

The effect on the nerves of these two bony overgrowths, the one in the modiolus filling the upper part of the internal auditory meatus, and the second resulting in the thickening of the periosteal layer must be harmful. The first squeezes the nerve; the second greatly lengthens the distance from the respective ganglia of the cochlear and vestibular division of the 8th nerve and thus stretches the nerve.

Compression not only of the cochlear but also of the vestibular division of the 8th nerve by new bone formed in and near the modiolus can be well seen in Fig. 12*a*, which represents the cochlea of a dog after 5 months on a vitamin A-deficient diet. In this figure a smaller mass of new bone (NB^1) is present in the modiolus above the spiral ganglion at the base of the helix and a second larger mass (NB^2) between the division of the cochlear and vestibular branches of the main nerve. The spiral ganglion cells have largely degenerated (see Fig. 7 and compare with the normal in Fig. 6), but in the case of the cells of Scarpa's ganglion, although many of these are squeezed into an abnormal shape, they have not degenerated (see Fig. 14 and compare with the normal in Fig. 13).

It has been mentioned above and elsewhere [Mellanby, 1935] that the cochlear division of the 8th nerve is more liable to be completely destroyed by these nutritional conditions than the vestibular division, and yet both branches are compressed and stretched by these bony overgrowths. This possible difference in susceptibility to mechanical influences will be considered later.

Serous labyrinthitis. Besides the nerve and bone changes described above, the condition known as serous labyrinthitis was also produced by these diets deficient in vitamin A. The albuminoid coagula in the perilymph spaces which are characteristic of this condition can be seen in Figs. 4 and 5. In the normal animal (Fig. 3) no such coagula can be seen. A similar pathological condition is well known in man. There is no evidence in the present experiments that the process is infective in origin, nor indeed that any of the changes described is the result of inflammation.

What the relation is between the serous labyrinthitis in the vitamin A-deficient animals and the nerve and bone changes cannot yet be stated. It is possible that the abnormality of the perilymph is due to the spread of some toxic influence from the sub-arachnoid space of the posterior fossa up the cochlear aqueduct, as this is a common pathway for the transit of toxins in serous labyrinthitis in man [Hallpike, 1937]. This point i being further investigated.

Serous labyrinthitis in man is also associated with a series of degenerative changes in the organ of Corti and the sensory epithelium of the vestibule. Similar changes take place in the animals under discussion.

Degenerative changes in the organ of Corti. In serous labyrinthitis in man, one of the earliest changes in the organ of Corti is the loss of differential staining power between cytoplasm and nuclei in the cells of the rods of Corti and in the outer and inner hair cells. This change cannot be seen in the histological preparations so far made in the present work, probably because the technique used is not suitable. In severer cases of experimentally produced serous labyrinthitis, the degeneration of these cells becomes more marked, the hair cells lose their hairy projections and ultimately these auditory cells, as well as Deiter's and other cells of the organ of Corti disappear, leaving a small amount of undifferentiated tissue lying on the basement membrane (Fig. 5). In spite of the degeneration of these cells, there still remains the small tunnel of Corti. In Fig. 3, representing the organ of Corti of a dog receiving vitamin A, the structure is normal.

Degenerative changes in the epithelial cells of the ampullae. Up to the present time a comparative study of the vestibular apparatus has not been made but changes in the epithelium of the semicircular canals in those animals fed for long periods on A-deficient diets are also obvious. Comparing Figs. 16*a* and 16*b* (vitamin A deficient) with Figs. 15*a* and 15*b* (receiving vitamin A), it will be seen that there is a general thickening of the epithelium in the former. Associated with this thickening there is a loss of differentiation of the individual cells, the disappearance of the nuclei and a shedding of hairy projections.

Relation of serous labyrinthitis to end-organ degeneration. When the degenerative changes in the organ of Corti and in the sensory epithelium of the vestibule above described were first noted, it was thought that these might be due to degeneration of the cochlear and vestibular nerves respectively. Dr Hallpike informed me, however, that this was not the case, since there was good evidence that cutting the 8th nerve, although it produced degeneration of the whole cochlear neurone, did not produce degenerative changes in the organ of Corti. He also informed me that serous labyrinthitis in man ultimately affected the organ of Corti and the sensory epithelium of the ampullae. It seems likely, therefore, that this is the sequence of events in the present experiments.

Serious structural changes in the organ of Corti are a late event compared with the nerve degeneration and bone hyperplasia and appear always to be accompanied by serous labyrinthitis. On the other hand, in shorter experiments there may be serous labyrinthitis without large structural defects in the organ of Corti. There is also a suggestion from one result in the present work that serous labyrinthitis can be cured by adding vitamin A to the diet even after the bone overgrowth and nerve degeneration are advanced. It is likely, for instance, by analogy with the other experimental results that dog III (curative experiment) had developed serous labyrinthitis as well as nerve and bone changes at the end of the 15 weeks on a vitamin A-deficient diet. This dog received abundant vitamin A for the next 27 weeks and at the end of this period the cochlear neurone had almost completely degenerated. There was, however, little or no serous labyrinthitis nor was there any abnormality of the organ of Corti or sensory epithelium of the ampullae of the semicircular canals. It would appear possible from this experiment that the addition of vitamin A, after a period of vitamin A deficiency, cleared up the serous labyrinthitis and so prevented the degeneration of the organ of Corti but did not prevent the process of destruction of the cochlear neurone. This possible explanation would be in keeping with the view that degeneration of the sensory epithelium of the labyrinth in these experiments is due to serous labyrinthitis and not to degeneration of the auditory nerve. Further experiments to settle this point are, however, necessary.

Serous labyrinthitis is probably not so early a pathological development as bone overgrowth or nerve degeneration, although it is true that wherever bone hyperplasia and nerve degeneration are advanced (apart from the curative experiment of dog III) serous labyrinthitis exists. In early and slighter cases of bone and nerve abnormality there may be no serous labyrinthitis. This is best seen in experiments in which the cereal element of a vitamin A-deficient diet is replaced by potato. The amount of bone hyperplasia in such cases is comparatively small though definite, and the cells of the spiral ganglion are slightly degenerated, but there is little or no serous labyrinthitis and the cells of the end-organs are apparently normal (Fig. 12*b*).

Early changes in labyrinth due to dietetic abnormality. As some of the results, especially the bony overgrowth, were quite unexpected, the appearance in earlier stages of the abnormality has not so far been studied, although histological material is now being prepared from experiments of shorter duration.

These early changes can, however, be well seen in a series of experiments in which the cereal portion of the diet was replaced by potato. The diets otherwise were as previously described. It has been already pointed out [Mellanby, 1934*b*] that replacement of cereal by potato in these vitamin A-deficient diets greatly reduces the degree of nerve degeneration. What may be the real explanation of this effect is not known—it is possible that cereals have a toxic action and hasten the pathological changes in the absence of vitamin A and that their replacement by potato reduces this action, or it may be that the small amount of carotene in potato is sufficient to prevent the development of the more pronounced changes. It may be added, however, that the dogs eating the potato diets had no stores of vitamin A in the liver at death. This point will not be considered further here.

After a period of 5 months on the potato diet deficient in vitamin, the animals were very active and comparatively normal in appearance and behaviour, and contrasted greatly with those on a similar diet but containing cereal. Histological examination of the labyrinthine capsule at the end of this period usually shows a small amount of bone overgrowth in the modiolus and increased periosteal bone; this can be clearly seen in Fig. 12*b*. How relatively small the bone overgrowth is can be seen if comparison is made with Fig. 12*a*, which represents the labyrinth of a dog maintained on a cereal diet deficient in vitamin A for the same period (i.e. 5 months) as the potato-fed dog of Fig. 12*b*.

The amount of nerve degeneration in this latter dog is slight but definite. There is actually only a small amount of degeneration in the cochlear neurone (central fibres) but the spiral ganglion cells appear abnormal to some extent.

Little or no serous labyrinthitis is present in these cases and no degenerative changes of the organ of Corti or the sensory epithelium of the vestibule can be seen.

Overgrowth of bone at the base of the skull obvious to the naked eye. After microscopic examination had revealed the large bony overgrowth produced by these vitamin A-deficient diets it was soon obvious, even to the naked eye, that the base of the skulls of these animals was greatly deformed by this overgrowth. In addition to the auditory nerve, it was evident at once that other nerves including the optic, trigeminal and facial were pinched by the bony overgrowth and that this mechanical pressure might well explain the degenerative changes of these nerves previously described. This matter will be considered in more detail in a subsequent publication.

Rabbits and rats. That there are also changes in rabbits and rats fed on vitamin A-deficient diets there can be no doubt, but details of the abnormalities have not been studied sufficiently to warrant a description here. In the vitamin A-deficient animals so far examined, there are abnormalities, including nerve fibre and cell degeneration, bony overgrowth and serous labyrinthitis.

DISCUSSION OF RESULTS

Similar experiments previously published [Mellanby, 1934*b*, 1935] had shown that the central branches of the cochlear and vestibular divisions of the 8th nerve in the labyrinthine capsule were degenerated to some degree, the cochlear fibres more than the vestibular. The present work confirms this earlier work and allows a much better judgment to be made both of the degree of degeneration and the condition of the various parts of the cochlear neurone. In some cases the whole cochlear division had degenerated and the animals must have been completely deaf. In such cases the vestibular division was also severely affected but not to the same degree, and even when the cells of the spiral ganglion had completely disappeared it was usual to find some cells of Scarpa's ganglion remaining.

The surprising result of the present investigation was the new bone formation in the labyrinthine capsule which accompanied the nerve degeneration. So far as the writer knows, there is no previous evidence that vitamin A deficiency results in bone overgrowth. This new bone, so far as the labyrinth is concerned, affects the periosteal bone near the brain and is not found in the deeper layers away from the brain. It is of cancellous nature with marrow cavities usually full of fat. It is normal in appearance and is not of inflammatory origin. Indeed, the capsules of these affected dogs are apparently free from infection in the ordinary sense. This point is mentioned because it will be remembered that rats brought up on vitamin A-deficient diets often have infected middle ears [Green & Mellanby, 1928].

It has been pointed out that the new bone laid down appears constantly in two places, although in each it is of periosteal origin. The first of these positions is in and near the modiolus itself. This bone may appear more compact and usually only one or two cavities, sometimes large, are obvious in any one section. This bone can be seen in the photomicrographs to press on and elongate the nerves as they leave the cochlea and vestibule respectively. The second position of bone overgrowth is round the internal auditory meatus and adjacent to the brain. It has the effect of placing the cochlea deeper into the capsule and of increasing the distance from the spiral and Scarpa's ganglia to the brain. This must stretch the nerve. In addition, however, this bony overgrowth sometimes twists the internal auditory meatus, so that it may be impossible to get a complete section of the nerve in one histological preparation, as can usually be done in normal animals. This twisting of the internal auditory meatus must also stretch the nerve.

New bone may also occasionally be seen in a third place, namely in the scala tympani at the basal whorl of the helix. The inconstancy of the small amount of bone in this third position indicates that it is probably independent of the other two masses of periosteal bone and may rather be related to serous labyrinthitis.

✓ The general result of this investigation, therefore, is that a deficiency of vitamin A in the diet has resulted in the development of newly formed bone and the degeneration of the 8th nerve, especially the cochlear neurones. As a rule, the greater the bone formation, the greater the degeneration of the nerves of the labyrinth, and the conclusion that the nerve degeneration is due to mechanical interference produced by bone overgrowth is difficult to avoid. This deduction is supported by what is already known of the reaction of each division of the auditory nerve to injury of their central branches. If the cochlear nerve reacted as do most sensory nerves to injury of its central branch, it would not be expected that the cells of its spiral ganglion and their peripheral branches should be so easily destroyed by mechanical influences bearing on its central branch in the internal auditory meatus. It is known, however, that the cochlear nerve is unlike most other sensory nerves in this respect and is even unlike the vestibular division. For instance, Wittmaack [1911] showed that destruction of the central branch of the 8th nerve in the internal auditory meatus with preservation of the blood supply caused degeneration of the cells of the spiral ganglion with their peripheral branches to the organ of Corti. Wittmaack also found that the peripheral vestibular branch and Scarpa's ganglion did not degenerate after this operative procedure, although the central branches of the vestibular nerve were destroyed. These results have been confirmed by Kaida [1931], by Hallpike & Rawdon-Smith [1934] and Hallpike [1938]. Other confirmation comes from the observations of Crowe [1929], corroborated by de Kleyn & Gray [1932], that pressure upon the 8th nerve by a tumour in the internal auditory meatus leads to degeneration of the peripheral cochlear neurone.

These facts show that the degenerative reactions of the cochlear nerve represent a notable exception to Waller's law. It is clear, also, that the condition of the cochlear neurone in the present work is in keeping with its degeneration being due to pressure and stretching of the central fibres by the newly formed bony masses.

In contrast to the great susceptibility of the cochlear nerve to compression and other injuries of its central branch, the resistance of the vestibular nerve to compression and elongation, which has been demonstrated above (Figs. 4, 5), is noteworthy. The experiments of other workers have also demonstrated this fact, for cutting the central branch of the vestibular neurone, as in the case of most other afferent nerves, produces only temporary derangement of Scarpa's ganglion and no degeneration of the peripheral branch [Hallpike, 1937]. Thus the better preserved condition of the vestibular nerve in comparison with that of the cochlear nerve again supports the view that the nerve destructive changes are mechanical in origin and are due to bone overgrowth.

The point under discussion, namely, whether bone overgrowth is directly responsible for the degeneration of the 8th nerve, is of fundamental importance and obviously cannot rest at the present point. The nerve degeneration produced by these vitamin A-deficient diets is widespread and includes not only the 8th nerve but also the optic and trigeminal nerves, the posterior roots of the spinal cord and many ascending fibres in the cord, including the endogenous nerve fibres of the anterior and posterior spino-cerebellar tracts. If, therefore, bone overgrowth is responsible for the death of the auditory nerve, it would be expected that the degenerative change in all the other nerves, peripheral and central, would have a similar cause. It is, therefore, desirable that the evidence that the auditory nerve has been killed in these experimental animals because of the overgrowth of bone should be as clear as possible, and that the possibility that the bone overgrowth is secondary to degeneration of the nerve or that both bone overgrowth and nerve degeneration are independent of each other but dependent upon some third factor should be excluded.

On the whole, the evidence of the present experiment that the overgrowth of bone of the labyrinthine capsule is responsible for the death of the nerves is as clear as can probably be expected. It is hoped that further experiments in which slight degrees of change are produced will help to settle the point at issue. Even so, however, the present results demanded that the investigation should be extended to see how the other nerves of the brain and spinal cord, which are also known to degenerate under these nutritional conditions, are affected by bone overgrowth in their immediate neighbourhood.

This work is now being done and it is already clear that there is much bone hyperplasia in the neighbourhood of the optic and trigeminal nerves. Indeed the skull in immediate contact with the base of the brain, including the bone surrounding the various foramina, is undoubtedly deformed in dogs brought up on these vitamin A-deficient diets. Early examination, therefore, supports the view that the degeneration of other nerves of the brain may well be due to the bone overgrowth as in the case of the auditory nerves. It would be expected, therefore, that a similar mechanism holds in the case of the nerves of the spinal cord, and further examination is being made on this point. It is difficult at first sight to see how mechanical factors due to bone overgrowth can destroy endogenous fibres of the central nervous system, such as those of the anterior and posterior spino-cerebellar tracts, but this point may be clarified by further investigation. In the meantime, so far as the present work goes, it seems certain that the degeneration of the auditory nerve brought about in young dogs by diets deficient in vitamin A and made up of ordinary foodstuffs is due primarily to excessive bone formation of the periosteal bone of the labyrinthine capsule and the mechanical interference of this new bone with the auditory nerve.

There remains for consideration the relation between serous labyrinthitis present in the vitamin A-deficient dogs and the bone and nerve changes above described. But little can be said of this at the present stage of the work. It is probable that serous labyrinthitis is not the earliest change, since in some dogs showing only slight abnormalities, as for instance in the animals receiving potato in their diet instead of cereal, although there is definite bone hyperplasia and early nerve degeneration, there is little or no serous labyrinthitis. In such cases albuminoid coagula are not obvious in the perilymph and the organ of Corti is normal in appearance. On the other hand, where the bone overgrowth and nerve degeneration are advanced, there is always serous labyrinthitis. It looks as if the bone and nerve changes precede the development of labyrinthitis in the earlier period of pathological change.

Another difference between the nerve degeneration and serous labyrinthitis is suggested by the appearance of the labyrinth of dog III (curative experiment). In this animal the cochlear neurones had completely disappeared, but there was no labyrinthitis. By comparison with other experimental animals it seems likely that this animal also had serous labyrinthitis at the end of the 15 weeks on a vitamin A-deficient diet. If this were the case, then the addition of vitamin A for the following 27 weeks must have cleared up the condition. It has not, however, prevented the nerve degeneration from going on to completion. Reasons have been given above for thinking that the degenerative changes in the organ of Corti and the sensory epithelium of the ampullae of the semicircular canals are associated with the development of serous labyrinthitis and are not related to the nerve degeneration. It was pointed out, for instance, that the cochlear neurone may have completely disappeared, and yet in the absence of serous labyrinthitis the organ of Corti may appear normal or nearly normal.

Finally it may be asked, how do these vitamin A-deficient diets produce all these changes in the labyrinthine capsule? This question cannot be answered at present, but it is already clear that the answer must include not only consideration of the auditory mechanism but of the many other nerves in the body. Although it is probable that overgrowth of new bone holds the secret to the sequence of pathological changes, in the cranial nerves at least, this part of the subject has not been sufficiently investigated to allow a full description to be given, except in the case of the auditory nerve. On the other hand, a fairly complete survey of nerve degeneration produced by these diets has been worked out in the case of the dog and rabbit [Mellanby, 1934*b*, 1935], and it is now necessary to see whether all such degenerative changes in the spinal cord as well as the brain can be related directly or indirectly to overgrowth and pressure of bone. Whether this is so or not, it is already evident that overgrowth of bone in young animals due to vitamin A deficiency does not affect all bones. In the long bones the absence of vitamin A probably affects the texture of the cancellous tissue but it does not appear to alter the shape of these bones.

In view of the historical development of knowledge of bone growth, especially in regard to rickets and the discovery of the calcifying vitamin D, it is of peculiar interest that vitamin A, with which vitamin D is so often associated, should also be concerned with bone formation. The parts played by these two vitamins are, however, essentially different. In the absence of vitamin D there is overgrowth of osteoid tissue in all growing bone. In the absence of vitamin A there is overgrowth of apparently normally formed bone in certain places. In view of the growth-promoting function usually assigned to vitamin A, it is of interest to note that its presence in young dogs *prevents* overgrowth of the labyrinthine bone. It would be fruitless at this stage to conjecture the nature of the mechanism whereby vitamin A works in limiting the growth of bones at the base of the skull and other places to their normal shape, so that in its absence these bones overgrow and destroy the nerves. It is certain, however, that the explanation of this mechanism must depend on interesting physiological processes, of whose nature nothing is known at the present time.

SUMMARY

1. The histological examination of the labyrinth capsules of young dogs fed for some months on diets of natural foodstuffs but deficient in vitamin A and rich in cereals revealed the following changes:

(*a*) degeneration of different degrees up to complete disappearance of the cochlear nerve, the cells of the spiral ganglion and their central and peripheral branches;

(*b*) degeneration, but to a lesser degree, of the vestibular division of the 8th nerve;

(*c*) overgrowth of bone in the modiolus and of the periosteal layer of the capsule near the brain.

2. This overgrowth of bone is apparently responsible for the degenerative changes in the nerves by reason of the pressing and stretching of these tissues.

3. Serous labyrinthitis also develops in the cochlea of the dogs on a vitamin A-deficient diet. This condition seems to produce degeneration of the sensory epithelium of the labyrinth, including that of the organ of Corti and of the ampullae of the semicircular canals, in course of time.

4. Substitution of potato for the cereal element of these vitamin A-deficient diets greatly reduces the abnormal changes in the labyrinth above described.

5. Examination of the base of the skull of these vitamin A-deficient dogs reveals other bone overgrowth and deformity, which is probably responsible for the degenerative changes of other cranial nerves, such as the optic and trigeminal nerves, previously described.

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EXPLANATION OF PLATES I–IV

PLATE I

Fig. 1. Low-power photograph (× 10) of cochlea of dog I. Basal diet with vitamin A added. On diet 10 months. It will be seen that the internal auditory meatus is clear and the cochlear branch of the 8th nerve (*N*) has an uninterrupted path to the brain. The spiral ganglion cells (*Sp*) are clearly seen (cp. Figs. 2, etc.).

Fig. 2. Low-power photograph (× 10) of cochlea of dog II. Basal diet as for dog I, but in this case no carotene or vitamin A was added. On diet 10 months. Two new bony masses (*NB*) practically filling the internal auditory meatus can be seen. The cells of the spiral ganglion (*Sp′*) have disappeared and are replaced by connective tissue. Albuminoid coagula (*C*) can be well seen in scala tympani, indicating serous labyrinthitis.

Fig. 3. Middle whorl of cochlea (× 27) of a normal dog showing organ of Corti and cells of spiral ganglion (*Sp*).

Fig. 4. Middle whorl of cochlea (× 27) of a dog on a diet containing much cereal and deficient in vitamin A. On diet 5 months. Many cells of the spiral ganglion (*Sp′*) have disappeared together with the peripheral branches to the organ of Corti. Indications of serous labyrinthitis can be seen in the scala vestibuli.

PLATE II

Fig. 5. Basal whorl of cochlea (× 27) of dog II. Diet deficient in vitamin A. On diet 10 months. The cells of the spiral ganglion (*Sp′*) have completely disappeared together with the peripheral nerve fibres to the organ of Corti. Albuminoid coagula (*C*) are obvious in both the scala tympani and scala vestibuli, indicating a condition of serous labyrinthitis. The organ of Corti is very abnormal.

Fig. 6. High-power photograph (× 266) of cells of the spiral ganglion of a normal dog. Note definite Nissl granules and well-defined nuclei.

Fig. 7. High-power photograph (× 266) of cells of the spiral ganglion of a dog whose diet was deficient in vitamin A. Nearly all the ganglion cells have completely degenerated and the few remaining cells are shrunken and the protoplasm is homogeneous. Cytoplasm is devoid of granules.

Fig. 8. Low-power photograph (× 3, 2) of the cochlea and labyrinthine capsule of dog I on a diet rich in cereals and containing vitamin A. Normal or nearly normal structure.

Fig. 9*a*. Cochlea and labyrinthine capsule (× 3, 2) of dog II on a diet rich in cereals and deficient in vitamin A. Not only is the internal auditory meatus nearly filled with new bone (*NB*), but the periosteal bone of the capsule, especially on the brain aspect, is greatly increased in thickness, the new bone being cancellous in type. The exit of the 8th nerve is not seen in this section.

Fig. 9*b*. The section represented here is about 2·5 mm. from that shown in Fig. 9*a*. The exit of the 8th nerve can now be seen. The two photographs (Figs. 9*a*, 9*b*) when compared with the normal (Fig. 8) illustrate the tortuous course, the compression and the stretching of the auditory nerve resulting from the bony overgrowth in dogs brought up on a vitamin A-deficient diet.

Plate III

Fig. 10. Labyrinthine capsule (× 3, 7) of a normal dog, but in a plane at right angles to Figs. 8, 9*a* and 9*b*.

Fig. 11. Labyrinthine capsule (× 3, 7) of a dog on a diet rich in cereals and deficient in vitamin A. There is again a mass of new bony growth in the modiolus (*NB*) and great thickening of the periosteal layer of the labyrinthine capsule, causing compression and elongation of the nerve. Compare with Fig. 10, a normal section cut in the same plane.

Fig. 12*a*. Bony overgrowth (NB^1 and NB^2) causing compression of both the cochlear and vestibular branches (*N*) of the 8th nerve in a dog on a cereal diet deficient in vitamin A (× 12). Only a few degenerate cells remain in the spiral ganglion but more are found in Scarpa's ganglion (see Figs. 13, 14).

Fig. 12*b*. Cochlea (× 12) of a dog maintained on a diet deficient in vitamin A for 5 months. The diet was similar to that of the previous case (Fig. 12*a*) except that potato replaced the cereal. The cells of the spiral ganglion are only slightly degenerate. There is some new bone in the modiolus (*NB*) and slight thickening of the periosteal bone. The changes seen in this animal on a potato diet are much less advanced than those of the animal on a cereal diet for the same period (Fig. 12*a*).

Plate IV

Fig. 13. Cells of Scarpa's ganglion (× 266) of a normal dog.

Fig. 14. Cells of Scarpa's ganglion (× 266) of a dog on a diet rich in cereals and deficient in vitamin A (see Fig. 12*a*). Although the cells are comparatively normal in structure, they are markedly elongated, apparently due to the pressure of the new bone (*NB*) shown in Fig. 12*a*. These cells seem very resistant to mechanical injury as compared with those of the spiral ganglion.

Figs. 15*a* and 15*b*. Low-power (× 27) and high-power (× 300) photographs of ampulla of dog I on a diet rich in cereals and vitamin A. The epithelium is thin, there is good differentiation between nuclei and cytoplasm of the cells and the hair-like projections are clearly seen.

Figs. 16*a* and 16*b*. Low-power (× 27) and high-power (× 300) photographs of ampulla of dog II on a cereal diet deficient in vitamin A. The epithelium is thickened, the differentiation between nuclei and cytoplasm is poor, and the hairy projections have in some places disappeared.

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THE RELATION OF TRANSIENT ORANGE TO VISUAL PURPLE AND INDICATOR YELLOW

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A SLIGHTLY alkaline solution of visual purple which is exposed to daylight at room temperature rapidly becomes pale yellow in colour. The yellow colour is due to the formation during bleaching of a substance which has indicator properties. This substance assumes a deep chrome yellow in acid and a pale yellow colour in alkaline solution. It will be referred to as "indicator yellow" [Lythgoe, 1937]. There is, however, an intermediate stage in the bleaching process, since if the original alkaline solution of visual purple is first cooled to 0° C. and then exposed to light, the solution becomes orange in colour. The orange colour fades slowly at 0° but very rapidly if the solution is allowed to regain room temperature (20° C.), and after it has faded the presence of indicator yellow can be demonstrated in the solution. The use of the term "transient orange" to describe the substance responsible for the intermediate orange colour was suggested in a previous paper [Lythgoe, 1937]. As Wald [1936] pointed out, it is never possible to regain the original orange colour of freshly bleached visual purple once the pure yellow of the indicator substance has made its appearance.

The purpose of this paper is to advance further evidence to support the view that visual purple is decomposed by light to form transient orange and that the latter substance is thermally converted to indicator yellow at a rapid rate.

Since the thermal reaction is rapid, it can be studied accurately only by the use of temperatures in the region of 0° C. As will be shown later, it is important to realize that the "dark reaction" may be made up of two changes, viz. the decomposition of transient orange, and the subsequent decomposition of the indicator yellow formed from it. Thermal changes of density after bleaching have been observed by many workers, notably by Hosoya [1933] and Wald [1938], but with the exception of Chase's results [1936] the solutions used have been either so warm or so acid that the destruction of transient orange must have been virtually completed before readings began. Provided only those solutions of visual purple containing a minimum of yellow impurities are used, the indicator yellow is stable on the alkaline side of neutrality [Lythgoe, 1937; Dartnall, Goodeve & Lythgoe, 1938]. The dark reaction in these solutions is composed entirely of the thermal decomposition of transient orange.

In the experiments to be described solutions of visual purple at a low temperature were bleached very rapidly by a strong light and the subsequent changes of density in the dark were followed at a number of wave-lengths.

APPARATUS AND MATERIAL

The optical densities were measured with a slightly modified form of the photoelectric spectro-photometer described by Bayliss, Lythgoe & Tansley [1936] (Fig. 1).

The light source of the photometer was a motor-car head-lamp bulb run off accumulators. The filament of the lamp was focused by a condensing lens through preliminary light filters [Lythgoe & Quilliam, 1938] on the entrance slit of the monochromator. The spectral band emerging from the exit slit of the monochromator, after being rendered parallel, was passed through the optical cell containing the solutions under examination. The capacity of the optical cell was about 0·5 c.c. and the length of path 0·5 cm.

The light transmitted by the solution falls on a photoelectric cell (Osram KG 7). The current thus generated is led to the insulated needle of a Lindemann electrometer. The potential on the needle is reduced to zero by inducing on it an equal and opposite charge through the condensers (C_1 and C_2). One plate of the condenser is connected both to the insulated needle and to the cathode of the photocell. The other plate of the condenser is connected to a potentiometer (*Pot.*) so that, when its potential is raised, the opposite plate is charged. With the earthing switch (*E.S.*) closed the needle is at earth potential and occupies its zero position. Just before the light shines on the cell the earthing switch is opened, and when the needle begins to move it is kept at zero by adjustment of the potentiometer. By means of a shutter the light is allowed to pass into the spectroscope for intervals of 5 sec. The final reading of the potentiometer is proportional to the quantity of light which has fallen on the photocell. By taking paired readings, one on a control optical cell filled with water and the other on the unknown solution, one can measure the fraction of incident light transmitted by the latter and so calculate its optical density. The sensitivity of the instrument was controlled both by using one or two condensers (C_1, C_2) and also by means of a two-gang selector switch which applied 1·5, 3·0, 4·5, 6·0, or 9·0 V. to the ends of the potentiometer.

The holder (*C.H.*) of the optical cell was hollow, and water was pumped through it to maintain the solution under examination at any desired temperature. In practice, three large reservoirs were used. One contained water at room temperature. The second was packed with ice and the water from the melting ice was pumped through the holder, maintaining the solution in the optical cell at about 2·9° C. The water in the third reservoir was kept at about 10° C. by a thermoregulator [Lythgoe & Quilliam, 1938].

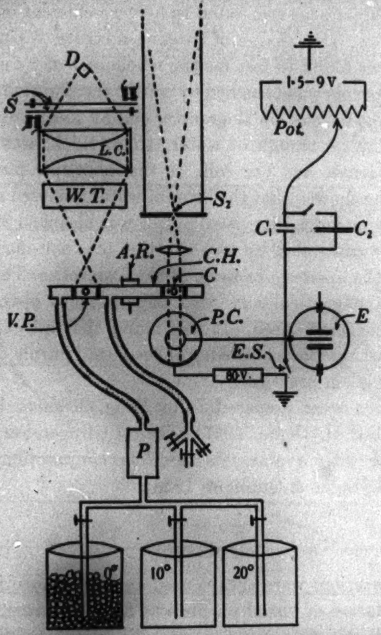

Fig. 1. Diagram of apparatus. *V.P.* solution under investigation; *C*, control solution; *C.H.* hollow cell holder; *A.R.* axis of rotation of cell holder; S_2, exit slit of spectroscope; *P.C.* photocell; *E*, Lindemann electrometer; C_1 and C_2, condensers; *E.S.* earthing switch; *D*, diamond of Pointolite; *L.C.* lantern condenser; *W.T.* water trough; *S*, blades of shutter and controlling electromagnets; *P*, water pump; 0°, 10°, 20°, the water reservoirs. The taps controlling the water circulation are also shown.

By means of two three-way taps, one in the outgoing and one in the returning water systems, it was possible to change quickly from one reservoir to another, so selecting the required temperature. A small glass ball was put into the optical cell and its movement on rotation of the cell holder served to stir the solution.

The formation of dew on the optical cell at low temperatures was prevented by blowing dry air on to the cell face and adjacent parts. The air was dried by passing it first through a metal spiral which was immersed in a freezing mixture, and secondly, through a Silica gel breather. This apparatus was found to be very efficient in preventing the formation of dew. One charge of freezing mixture (3 parts crushed ice: 1 part NaCl) was found to last for the whole of 1 day's experiments.

The visual purple was bleached by a 1000 C.P. Pointolite, the diamond (*D*) of which was focused on the optical cell by means of a large lantern condenser (*L.C.*). A trough of water, 4 cm. thick, was interposed between the condenser and the cell. A thermocouple placed in the cell during bleaching showed that the layer of water removed all the heat rays from the bleaching light. The visual purple was bleached for an accurately timed exposure controlled by two shutters (*S*), one made to open, the other to close the aperture in front of the Pointolite. The shutters were released electromagnetically by two trip switches operated by a disc rotating at a constant speed. It was found that an exposure of 1 sec. produced almost complete bleaching of the visual purple, but an exposure of 3 sec. was used in practice.

The solutions were prepared from *Rana esculenta* by the method already described [Lythgoe, 1937]. Their hydrogen-ion concentrations were measured with a glass electrode in conjunction with a valve electrometer [Lythgoe & Quilliam, 1938].

METHOD

Fresh preparations were always used and the absorption curve of the unbleached solution of visual purple was first measured. The holder for the optical cell was then rotated into the bleaching position and the solution was exposed to the light from the Pointolite for 3 sec. The cell holder was immediately rotated to the estimating position and a density measurement made. Each reading on the solution was paired with a control reading on an optical cell filled with distilled water. Usually the control followed immediately after the reading on the solution, but when it was desired to follow quick changes at several wave-lengths a set of control readings was taken at these wave-lengths both before and after the readings on the solution. The response of the apparatus was found to change very little over a period of 5 min. or so. After bleaching, density readings on the solution were then made in rotation at the wave-lengths under investigation until the end of the experiment.

Results

In one experiment (Fig. 2) the solution (pH 7·13) was cooled to 3° C. and the absorption curve of the unbleached visual purple was determined. The solution was then bleached, and density readings were taken in the range 460–490 mμ. The order in which readings were taken is indicated by the arrows in Fig. 2. The readings at 470, 480 and 490 mμ. were taken at 29, 44 and 59 sec. respectively after the end of bleaching, but the dark reaction is so fast, especially in its early stages, that there had already been a considerable loss of density before readings were begun. After readings in the range 460–490 mμ. had been repeated at intervals for about $\frac{3}{4}$ hr. the temperature of the solution was raised to about 20° C. for 20 min. and again cooled. The curve so obtained is marked *I.Y.*,

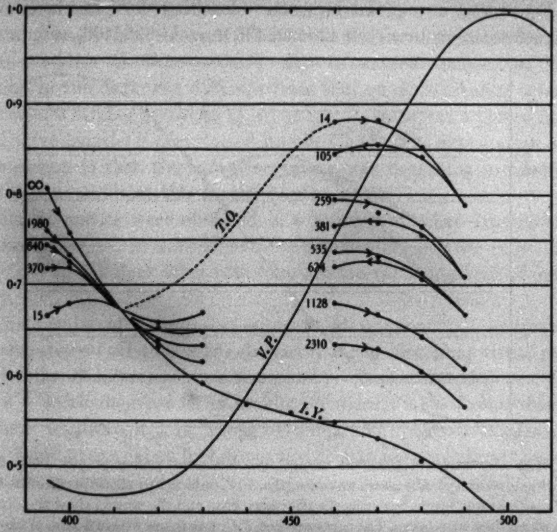

Fig. 2. The conversion of transient orange into indicator yellow. pH 7·13. 3° C. Ordinate: density. Abscissae: wave-lengths. *V.P.* the unbleached visual purple. The numbers against the points give the times (sec.) at which the readings were taken; the arrows show the direction in which they were taken. The readings on the curve marked *T.O.* were taken within $1\frac{1}{2}$ min. after bleaching. After the solution had been at 3° for 2500 sec. it was warmed to 20° for 20 min. and cooled again. The curve marked *I.Y.* was then taken. The graph embodies readings on two identical solutions.

and it will be seen that there has been a further loss of density. This is the absorption curve of indicator yellow and we know from other experiments that there would be only small further changes in density on keeping the solution.

After the completion of this run the cell was refilled with unbleached visual purple from the same preparation and bleached as before, but density readings were now taken in the range 395–430 mμ. It will be seen from Fig. 2 that during the dark after bleaching there has been a progressive decrease in density at 420 and 430 mμ. At 395, 400, and, to some extent, at 410 mμ., however, there has been an increase in density in the dark. At about 412 mμ. the density must have remained unaltered during the dark reaction. The increase in density at 395 and 400 mμ. is of importance in the interpretation of the changes which occur during the dark reaction.

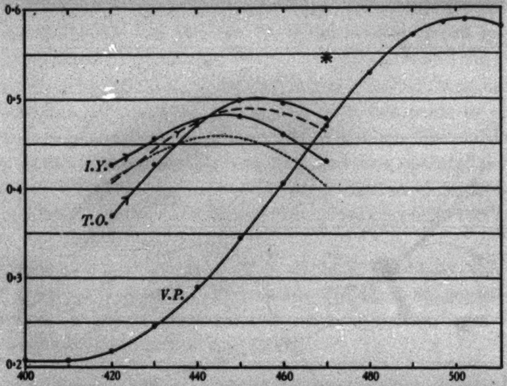

Fig. 3. The conversion of transient orange into indicator yellow at *p*H 4·90. 2·9° C. Ordinate: density. Abscissae: wave-lengths. *V.P.* unbleached visual purple. The taking of readings was begun immediately after bleaching and the arrows show the direction in which they were taken. The curve marked *T.O.* was taken within 3 min. of bleaching but it is not a true curve of transient orange. The asterisk records the density at 470 mμ. of an identical solution 10 sec. after bleaching. The readings for the upper dotted line were begun 270 sec. after bleaching. The curve marked *I.Y.* taken 1 hr. after bleaching is not very different from the true curve for indicator yellow. The lower dotted line represents readings taken 2½ hr. after bleaching; it records the decomposition of indicator yellow.

Fig. 3 shows the results of an experiment on a solution of visual purple at *p*H 4·90. The procedure was similar to that in the first experiment except that the range of wave-lengths was different. After bleaching, the densities in the dark decreased between 450 and 470 mμ. but increased between 420 and 440 mμ. The density at about 443 mμ. must have remained constant during the dark reaction. As with the experiment at *p*H 7·13, there had been a considerable loss of density before the first of the readings at 470 mμ. was taken. The asterisk in Fig. 3 marks the density at this wave-length 10 sec. after bleaching in another sample of the solution at *p*H 4·90. The curve marked *I.Y.* was taken 1 hr. after bleaching, and it is not very different from the curve for indicator yellow at this acidity [Lythgoe, 1937]. The subsequent behaviour of this solution in the dark was different from that of more alkaline solutions, since after a further period of 1⅓ hr. at 2·9° C. there was a loss of density at all wave-lengths (lower dotted line in Fig. 3). The explanation of this phenomenon is that the indicator yellow was beginning to decompose since it is unstable at this acidity [Lythgoe, 1937].

Similar results were obtained in a number of other experiments at other acidities, but in markedly acid or alkaline solutions the early changes of density in the dark after bleaching are too rapid to admit of detailed study.

The results of several experiments at a variety of hydrogen-ion concentrations (*p*H 4·90–9·25) showed that, on bleaching visual purple, the density at 470 mμ. shows a marked increase whilst that at 480 mμ. shows a small decrease. The maximum density of a freshly bleached solution is probably at about 470 mμ. The curves marked *T.O.* (Figs. 2 and 3) are only a first approximation to the true absorption curve of transient orange.

Discussion

When visual purple is bleached by light at room temperature the indicator yellow formed is relatively stable provided the solution is on the alkaline side of neutrality. The intermediate substance, transient orange, might be related to these substances in two ways. It might be a true intermediate substance, thus:

$$V.P. \xrightarrow{\text{light}} T.O. \xrightarrow{\text{thermal}} I.Y. \qquad \text{......(i)}$$

On the other hand, both indicator yellow and transient orange might be formed simultaneously, the latter then decomposing, thus:

$$\begin{tikzcd} & I.Y. & \\ V.P. + \text{light} \arrow[ur] \arrow[dr] & & \\ & T.O. \arrow[r, "\text{thermal}"] & \text{colourless breakdown products.} \end{tikzcd} \qquad \text{......(ii)}$$

If the latter were true then with the disappearance of transient orange there would be a progressive loss of density at all wave-lengths in the dark, the loss being greatest at the wave-length of its greatest density. The increasing density in the dark at the shorter wave-lengths is very strong evidence that another substance is being formed during the dark reaction. It will be shown later that the indicator reaction develops in the solution only as the orange colour disappears, and scheme (i) is therefore the more likely to be true. It would appear therefore that at *p*H 7·13 transient orange has a maximum absorption at about 470 mμ., whilst the indicator yellow formed from it has a maximum absorption in the near ultra-violet with a decreasing absorption towards the longer wave-lengths [Krause & Sidwell, 1938]. At about 412 mμ. the densities of transient orange and indicator yellow have the same value, and no matter what their relative proportions at any time and in any one solution, the density at that wave-length will be unaltered. During the dark reaction therefore we find an increase in density at wave-lengths shorter than 412 mμ., a decrease in density at wave-lengths longer than 412 mμ. with a maximum loss at about 470 mμ., whilst at the cross-over wave-length of 412 mμ. there is no change in density.

It has been shown that, in weakly acid solutions, indicator yellow is unstable, and that a cross-over wave-length is observed only during the early stages of the dark reaction. We also find that only those preparations of visual purple which are relatively transparent to the blue wave-lengths give clear-cut results, the impure solutions giving a gradual loss of density at all wave-lengths similar in appearance to the destruction of indicator yellow in acid solution. In such solutions the dark reaction is made up of (1) the conversion of transient orange into indicator yellow which we regard as the true dark reaction, (2) the decomposition of the indicator yellow formed. Both reactions probably occurred simultaneously in the experiments recorded by Hosoya [1933] and Wald [1937].

Wald followed the dark reaction at 29·3° C. (*p*H 6·9). He appears to have obtained a slight increase in absorption below 435 mμ. with a maximum of absorption at 480 mμ. His absorption curve taken 1·2 min. after bleaching is very much lower than ours taken at the same time interval, and it seems probable that most of the transient orange had already been decomposed at the high temperature used. For the most part the bleached solutions of Wald and of Hosoya showed a slow fall in density at all wave-lengths and in our view this reaction was predominantly due to the decomposition of indicator yellow.

Recently Wald [1938] has published results to show that in acid solution (pH 3·9 and 5, and to some extent at pH 6·9) there is an increase in density during the dark reaction which is greatest at 440 mμ. We have never found any trace of this rise in the few experiments we have made in this range of acidities and we feel that our failure to find it must be due to some fundamental difference between our solutions and those used by Wald. It should be emphasized however that we invariably worked at temperatures below 3° C., and that at the temperature used by Wald (25° C.) the reactions recorded in the present paper would have been practically completed before he had taken his first reading.

The term visual yellow is used to describe all the coloured breakdown products of visual purple. As we have seen, the term really embraces two substances which we call transient orange and indicator yellow. Are there other links in the chain? Unless there is an extremely rapid change before the appearance of transient orange, it would seem that so far as the visible spectrum is concerned there are no further links. From Fig. 2 it will be clear that since there is a single cross-over wave-length between the indicator yellow and the substance which is formed initially on bleaching, any further intermediate substance would have to possess the same density at that wave-length. Furthermore, the greatest loss of density occurs at about 470 mμ. during the whole of the true dark reaction. If two substances were concerned it would be unlikely that their absorption curves would be identical. The problem can be investigated quantitatively. If the fading of the orange colour in the dark involves nothing more than the quantitative conversion of transient orange to indicator yellow, then the progressive losses or gains occurring at one wave-length should be proportional to the losses or gains in density at any other wave-length. In other words, if we plot densities at one wave-length against the densities recorded at the same times at another wave-length, the points should fall on a straight line if the fading in the dark involves only a single chemical change. In Fig. 4 the densities at all the wave-lengths investigated (ordinates) have been plotted against the densities at 460 mμ. at the same times (abscissae). Actual density readings at 460 mμ. for all the times involved were not available but they were obtained by interpolation from a graph relating time with density at 460 mμ. This wave-length was chosen because we had many well-spaced readings for it. It will be seen that the points fall on straight lines within experimental error. This seems to be conclusive evidence that transient orange is a single absorbing molecular species and that its conversion to indicator yellow is the only reaction during the true dark reaction which can be studied in the visible spectrum.

The absorption curve of transient orange is not very markedly affected by hydrogen-ion concentration. This can be shown by the following test-tube experiment. Two samples of visual purple at about *p*H 5·0 were cooled to 0° C. and bleached side by side in daylight. Both samples turned orange. On making one sample alkaline (about *p*H 8·5), there was only a slight change of colour to a slightly more orange hue than that of the acid sample. This experiment is difficult to perform satisfactorily, since if a solution is ever either too acid or too alkaline the transient orange is very rapidly decomposed. The indicator reactions of transient orange must be much less marked than those of indicator

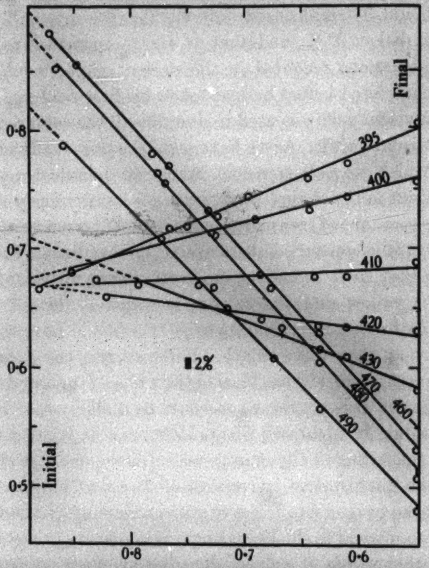

Fig. 4. The readings are the same as those shown in Fig. 2. Ordinate: density readings at a number of wave-lengths (395, 400, 410, 420, 430, 470, 480, 490 mμ.). Abscissae: the density readings at 460 mμ. at corresponding times (interpolated). The points lie on a series of straight lines within experimental error. (The short thick vertical line represents the extent of the displacement which would have occurred with a 2% error in the measurement of light transmissions.) The figure shows that the loss or gain of density at any wave-length is strictly proportional to the loss of density at 460 mμ.: the conversion of transient orange to indicator yellow is a simple chemical change.

yellow. This can be shown by another test-tube experiment. One sample of a preparation of visual purple at *p*H 5·0 was cooled to 0° C.; another sample remained at 20° C. On bleaching side by side in daylight the cooled sample turned orange, whilst the warmer one turned deep pure yellow. Equal volumes of an alkaline solution were added to each test-tube, but whereas the warmer sample gave the typical indicator reaction and turned a pale yellow, the cooled sample was very little affected, showing that if any change with altered acidity had occurred it was comparatively slight. Furthermore, there could not have been any considerable quantities of indicator yellow in the solution otherwise its colour change would have been seen. When the solutions were again made acid the warmer sample regained its original deep yellow colour but the cooled solution again remained unchanged. When the latter solution was warmed it became yellow in colour, and exhibited typical indicator properties.

Several experiments were performed in which the density of a visual purple solution was measured immediately after bleaching either at 470 or at 480 mμ. Within the *p*H range 4·9–9·25 there was always an increase in density at 470 mμ. and a small decrease in density at 480 mμ. This means that at some wave-length between 470 and 480 mμ. the density of the freshly bleached solution is identical with the density of the unbleached solution at the same wave-length. Since the absorption curve of visual purple at these wave-lengths is unaffected by hydrogen-ion concentration, it follows that the absorption curve of transient orange is not very markedly affected by this condition. Any effect which may exist is small compared with the acid-alkali reaction of indicator yellow. Strict experimental verification will be difficult, since we find that the conversion of transient orange is markedly accelerated in acid or alkaline solutions. A few seconds must elapse before the first reading can be taken on a freshly bleached solution, and during this time the amount of transient orange converted to indicator yellow will vary considerably in solutions of different acidity. The fact that transient orange has an absorption curve which is largely independent of hydrogen-ion concentration shows that it is structurally different from indicator yellow.

The results from an investigation now in progress on the influence of temperature and acidity on the decomposition of transient orange also show that this substance is quite different both from visual purple and from indicator yellow.

SUMMARY

1. Visual purple solutions were rapidly bleached in an intense light and the changes in density—the "dark reaction"—were followed at several wave-lengths.

2. The substance first formed by the action of light (transient orange) has an absorption equal to that of the parent visual purple at a wave-length between 470 and 480 mμ. The initial product of bleaching is very little affected by hydrogen-ion concentration.

3. In a solution at *p*H 7·13 the density at wave-lengths longer than 412 mμ. decreased during the dark reaction with a maximum loss in the region of 470 mμ., but at wave-lengths shorter than 412 mμ. the solution increased in density showing that another substance was being formed (indicator yellow).

4. These changes correspond to the conversion of unstable transient orange to indicator yellow which is a comparatively stable substance in alkaline solution.

5. The early changes in a more acid solution (*p*H 4·90) are similar, but subsequently the absorption curves show that the indicator yellow begins to decompose. The dark reaction is therefore composed of two separate reactions: (i) the thermal conversion of transient orange into indicator yellow—the true dark reaction, (ii) the thermal decomposition of indicator yellow to colourless products.

6. Evidence is advanced to support the view that transient orange is a distinct molecular species.

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CONCENTRATION AND SEDIMENTATION RATES OF BLOOD FROM THE SPLENIC ARTERY AND VEIN

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It was observed by Gawrilow [1928] that splenic vein blood in some instances sedimented more rapidly than blood from the general circulation, and in other cases more slowly. Berglund [1930] found that the sedimentation rate of splenic vein blood of horses was very much less than that of the artery. Stephens [1938*a*] observed a progressive diminution in the sedimentation rate of successive samples taken from the splenic vein when the splenic artery was clamped.

Sedimentation rates are greatly influenced by the red cell concentration of the blood, the greater the number of red cells per unit volume the less the sedimentation rate, other factors being equal. Now blood in the spleen pulp is more concentrated in red cells than that in the general circulation [Cruickshank, 1926; Feldberg & Lewin, 1928; Barcroft & Florey, 1928]. Moreover, it was found in the present experiments that the spleen may concentrate with great rapidity the blood supplied to it. This factor must therefore be considered first.

Immediate concentration of blood by the spleen

The spleens of cats were emptied via a cannula in the main splenic vein, then allowed to refill with the vein cannula closed, and the splenic artery clamped when the filling was complete. The subsidiary veins and arteries were ligated. The filling process required from 3 to 5 min. By faradic stimulation of the splenic nerves or of the spleen itself the contents were then expelled and the sequence of haematocrit values of splenic vein blood compared with that of the artery. The samples were taken in a 1 c.c. syringe graduated to 0·01 c.c., the blood being diluted with one-quarter its volume of 5% sodium citrate. Haematocrit values were determined on 100 mm. columns of citrated blood in Wintrobe tubes, all tubes being centrifuged together under standard conditions.

Table II shows typical haematocrit values for samples obtained in 5 min. from a cat spleen which required 4 min. to fill under nembutal anaesthesia.

TABLE I

	mm.
Splenic artery	28·5
1st splenic vein sample	17·0
2nd ,,	29·0
3rd ,,	33·5

The low haematocrit value of the first splenic vein sample is evidently due to corpuscles being retained in the spleen and released in the later samples. For another cat Fig. 3 (lower photograph, tubes *a*, *d*, *e*, *f* and *g*) shows the haematocrit values obtained in a similar experiment. Such results were obtained in seven cats and three dogs under anaesthesia, by nembutal, ether or urethane (two cats only).

Apparently the concentration of the blood occurs chiefly in the first 2 or 3 min. during filling. In fact, the overflow from the first refilling after emptying of cats' spleens could be seen with the naked eye to be dilute in red cells. When the spleen had been at rest untouched for some time this blood impoverished in red cells had evidently moved on.

Another mechanism by which the spleen concentrates blood by removing fluid via the lymphatics, described by Barcroft & Florey [1928], did not seem to be the important factor in the present experiments. Only by supposing that the spleen had retained the red cells and allowed the plasma to pass on can the haematocrit value of the first splenic vein sample, much lower than that of the artery, be accounted for. Moreover, concentration due to lymphatic drainage would seem to be excluded by the following further experiments. The spleens of cats were first emptied and refilled, the entire splenic mesentery, including artery, vein and lymphatics, then clamped, the splenic vein cannulated and samples withdrawn. These samples showed the same progression of haematocrit value with an initial value much below that of splenic arterial blood. The same precautions were observed in centrifugation. The errors of haematocrit determinations, admittedly rather large, could not account for the phenomena observed.

This latter method of performing the experiment affords the easiest method of demonstrating the effects.

Fig. 1 shows the haematocrit values of splenic vein blood from another type of experiment in cats, the splenic artery being untouched, a cannula placed in the splenic vein and samples taken from this during stimulated contraction of the spleen and during a phase of passive dilatation. Contraction and dilatation were caused to occur as rapidly as possible, and in the example shown 3–5 min. elapsed between each sample. All the samples were centrifuged together. The maximum error in haematocrit estimations of this sort is of the order of 1 mm. as determined by successive estimations on the femoral vein.

Rapid concentration of the blood by arrest of the corpuscles during filling of the spleen affords part of the explanation of the fact that whilst Cruickshank [1926] found that the red cell concentration of splenic vein blood showed first an increase and then a decrease as successive samples were taken, Feldberg & Lewin [1928] found no such variation. The time interval between the filling of the spleen and the taking of the blood samples is evidently a factor here. Fig. 2 shows the rise and fall referred to by Cruickshank, but in Figs. 3 and 5 a rise only is shown. In yet other experiments the progression was the inverse of that described by Cruickshank, with lower haematocrit values in the middle samples than at either the beginning or the end. The lower haematocrit value of terminal samples, when found, is probably due to the fact that the discharges from different regions of the spleen do not reach the vein simultaneously but in succession, the terminal discharge from one segment being followed by the initial discharge from another segment. Splenic innervation is segmental [Tait & Cashin, 1925].

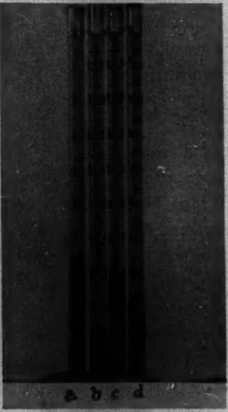

Fig. 1. Immediate concentration of blood by the spleen. *a*, spleen contracting; *b*, dilating; *c*, contracting; *d*, dilating. 3–5 min. intervals.

A time lag of 3 or 4 sec. between the moment of releasing a clip on the splenic artery and the commencement of flow in the vein was noticed when the spleens of cats were in the contracted state. The flow gave the impression of fluid percolating through a sponge.

SEDIMENTATION RATES OF BLOOD FROM SPLENIC ARTERY AND VEIN

Methods

Experiments in which the sedimentation of splenic arterial blood was compared with that of successive samples taken from a cannula in the main splenic vein, the entire splenic mesentery being clamped, were performed in twenty-six cats and seven dogs. By faradic stimulation of the splenic nerve or of the spleen itself the terminal contents of the spleen were disgorged, after the initial free flow had ceased. Clamping of the splenic artery is necessary as Cruickshank [1926] showed to prevent mixture of splenic pulp blood with that by-passed directly from the artery. Accordingly, the entire mesentery was clamped in order to close all the arteries and veins simultaneously. A useful point of technique for experiments of this and other types described, is to clamp the entire splenic mesentery with a long rubber-jawed bowel clamp before the vein samples are taken. Further entry of blood from small adventitious arteries is thus avoided.

The blood samples were obtained by withdrawal into sodium citrate as already described for the blood concentration experiments. Sedimentation was observed in Wintrobe tubes 100 mm. in length and 2·5–3 mm. bore. Haematocrit values were obtained by centrifuging the same specimens in the same tubes upon which sedimentation rates were observed, the tubes being centrifuged together. The tubes were filled by pipetting from the collected samples, and it was arranged that the inside wall of the tube above the column was wet with the blood. These factors of technique influence sedimentation rates.

The effects observed were independent of the type of anaesthesia, except that when ether was employed the spleen contained so little blood that it was necessary first to fill it by pressure on the splenic vein. Nembutal gives at laparotomy a dilated spleen engorged with blood; urethane gives less engorgement; with ether it is usually contracted.

Results

These were as follows:

(*a*) In blood from the resting spleen there was a much lower sedimentation rate in the splenic vein than in the artery, and also a small progressive decrease in the sedimentation rate as the later vein samples were reached [Stephens, 1938*a*].

(*b*) When the spleen had been recently refilled with blood the progressive decrease in the sedimentation rate of the later vein samples became extremely marked. Immediately after refilling, moreover, the initial vein samples had a sedimentation rate much greater than that of the artery.

(*c*) When the spleen was emptied and refilled and then left for some time with the vein alone clamped, the results were indistinguishable from those found with resting spleens.

In two cats the first vein sample obtained showed a lesser sedimentation rate than the artery, followed by a rate greater than the artery and then by a progressively lesser rate. Pressure on the spleen followed by refilling probably accounted for this sequence, an explanation in accord with the corresponding haematocrit values.

The general result obtained on resting spleens is typified in Fig. 2. In dogs the normal blood sedimentation rate is from 0·5 to 1·5 mm./hr., but the splenic vein sedimentation rate is so low that several hours are necessary before any measurable sedimentation can be seen. After 4 hr. the arterial blood sedimented 5 mm. in this case, whilst the vein blood was quite unsedimented. The haematocrit values of the same blood specimens is also shown in Fig. 2. (The photograph of these haematocrits was unavoidably delayed in this case so that some evaporation has occurred.) The maximum haematocrit value in the splenic vein was, as shown, almost exactly twice that in the artery, viz. 41 and 79·5 mm. respectively.

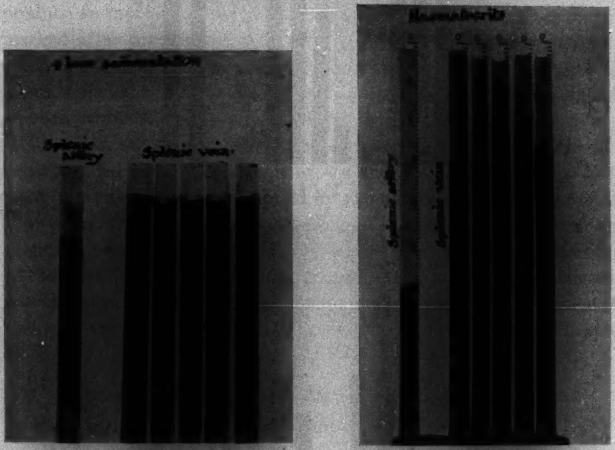

Fig. 2*a*. Fig. 2*b*.

Fig. 2. Resting spleen. Normal dog. Haematocrits of same specimens as 4 hr. sedimentations. No measurable sedimentation of splenic vein blood in this time. (Nembutal anaesthesia.)

It is possible that the results mean nothing more than that splenic pulp blood becomes increasingly more concentrated in erythrocytes as the deeper samples are reached. A reciprocal relationship between sedimentation rate and haematocrit values for splenic vein blood was found in every case, well within the limits of accuracy of sedimentation rate and haematocrit measurements. Anomalies occurred, however, when the values for splenic vein blood were compared with those of the artery.

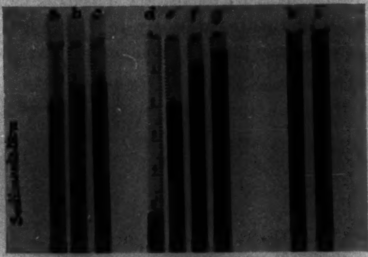

Fig. 3*a*.

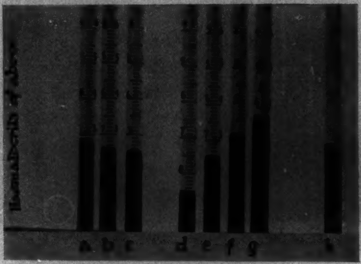

Fig. 3*b*.

Fig. 3. Immediately after refilling spleen. Pregnant cat. 2½ hr. sedimentation of: *a*, splenic artery at time of filling spleen after emptying; *d* to *g*, splenic vein samples taken in quick succession, artery clamped; *h* and *i*, foetal blood; *b* and *c*, splenic artery ½ and 1 hr. later showing effect of continued urethane. Below: haematocrits of same tubes, *i* removed. Sedimentation tubes set up from *i* to *a* at approximately 1 min. intervals.

ANAESTHESIA AND SEDIMENTATION RATE

Fig. 3 shows the effect of urethane anaesthesia on the sedimentation rate of cat blood from the splenic artery. There is a progressive decrease from the normal value (*a*), to (*c*), that after 1 hr. anaesthesia. Ether gave a similar result. Similar effects of chloroform anaesthesia have been observed by Rourke & Plass [1928] and with various anaesthetics by

Hino [1934]. In the case of ether and urethane it was not possible to explain these effects in the present experiments on the basis of increased red cell concentration of the peripheral blood. The haematocrit value became less as the anaesthesia proceeded, and yet the sedimentation rate decreased. Chloroform is known to produce a diminution of the blood sedimentation rate due to interference with fibrinogen production by the liver [Rourke & Plass, 1928].

In this connexion brief mention may be made of the fact that ether or chloroform and ether anaesthesia of cats and dogs was observed to produce an increase in red cell electrophoretic speed which became very marked after prolonged anaesthesia. Under nembutal or urethane the speed was practically unchanged. The electrophoretic speed and sedimentation changes under anaesthesia are evidently related to each other, and are probably due to altered plasma composition.

In order, therefore, to eliminate any effect due to the anaesthetic not being applied equally to splenic artery and pulp blood, in some experiments the spleen was first emptied by stimulation of the nerves and then allowed to fill with blood corresponding to the first splenic artery sample. Identical results were obtained. Progressive effects of the anaesthetic on the spleen pulp blood was, of course, improbable after the splenic artery was clamped. Moreover, the effects of anaesthesia on the sedimentation rate were very much smaller than the marked changes observed in splenic blood.

EFFECT OF REFILLING, PLASMA ALTERATION AND ANAEMIA

Four variations of the experiment were next performed:

(1) The splenic vein samples were taken immediately after emptying and refilling, and after the spleen had been left refilled for periods up to 1 hr.

Sampling immediately after refilling

When the initial splenic vein sample was taken immediately after refilling it showed a higher sedimentation rate than the splenic artery. (The two exceptions, already mentioned, were probably due to manipulation of the spleen.) This initial high rate is shown in Fig. 3. The corresponding haematocrit values showed that this was due to red cells being retained in the spleen and released in the later samples.

Sampling delayed after refilling

There were no significant differences when the blood was left in the spleen for 1 hr. with the entire splenic mesentery clamped. The initial high sedimentation rate, low haematocrit value and progressive sequence were found as before. It was desirable, however, that these experiments of delayed sampling after refilling should be extended with precautions to ensure that the lymphatic drainage was free.

Accordingly in four cats the spleen was emptied by stimulation, the principal vein alone ligated, and the spleen then left for periods from ¾ to 1 hr. During this time the lymphatic drainage was presumably free, and, what is perhaps equally important, the spleen pulp was exposed to the arterial blood pressure.

Marked differences were then found between the splenic vein samples thus obtained and those immediately after refilling. The results were now indistinguishable from those seen when the spleen had been at rest for long periods, resembling those shown in Fig. 2. The splenic vein haematocrit values were all approximately double that of the artery, whilst their sedimentation rates were all much less than that of the artery without any marked characteristic sequence.

Table II shows typical values after the vein had been ligated for 45 min. in a cat under nembutal.

TABLE II

	Haematocrit mm.	Sedimentation rate (1 hr.) mm.	Sedimentation rate (5 hr.) mm.
Splenic artery	26·5	23	42
1st splenic vein	50	less than 0·5	1·0
2nd ,,	50	less than 0·5	1·5
3rd ,,	46	less than 0·5	3·0
4th ,,	47	less than 0·5	3·0

Three factors must be considered in accounting for this effect:

(*a*) Lymphatic drainage causing further concentration of the blood.

(*b*) Drainage of blood impoverished in red cells via the small accessory veins which were not ligated.

(*c*) Red cells retained in the deeper regions of the spleen pulp may have moved on, perhaps aided by the pulsations of the spleen [Roy, 1881], and the arterial pressure. In any case, this would seem to be the most probable explanation of the marked increase in concentration of the first vein sample.

In order, therefore, to ascertain the part played by lymphatic drainage, it is necessary to ligate the numerous small veins in the splenic mesentery without damage to the lymphatics. This work is proceeding.

It is significant that persistent contraction of the splenic vein could cause concentration of the entire contents of the spleen by a mechanism similar to that involved in this experiment of ligating the main splenic vein alone. The marked contractility of this vessel, and its persistent contraction for an hour after stimulation, have been described by Tait & Cashin [1925].

(2) In cats with initial high blood sedimentation rate due to pregnancy the splenic vein samples collected immediately after filling the spleen showed the same progression. Fig. 3, upper photograph, shows the sequence for 2¼ hr. sedimentation in a pregnant cat. In this experiment the entire splenic mesentery was clamped after the spleen had refilled following emptying by faradic stimulation, the blood samples collected and set up as nearly as possible at the same time, but commencing with the tubes on the right-hand side of the photograph. The sedimentation differences shown are therefore rather less than those actually occurring.

The 1 hr. values in this case are shown in Table III.

TABLE III

	Sedimentation rate (1 hr.) mm.	Haematocrit (citrated blood) mm.
Splenic artery	6	27·5
1st splenic vein sample	23	14
2nd ,,	7·2	25
3rd ,,	2·5	30·5
4th ,,	1·5	36·5
Foetal blood	less than 0·5	28
Splenic artery ½ hr. later	5	25
Splenic artery 1½ hr. later	4·5	24·5

The first splenic vein sample after refilling has thus nearly four times the sedimentation rate of blood from the splenic artery, whilst the last vein sample has only one-quarter the sedimentation rate of the artery. The above table also shows the effect of urethane anaesthesia (no ether was used) and the reciprocal relationship between sedimentation rate and haematocrit value for splenic vein blood. Tubes *h* and *i* in Fig. 3 also show the extremely low sedimentation rate of foetal blood. Tube *i* was placed aside and observed for 8 hr., in which time the blood sedimented 3·5 mm. only. A similar result was obtained in other specimens of cat foetal blood, and by analogy with human foetal blood may be ascribed to its low plasma protein content.

(3) The sedimentation rate was artificially raised by intravenous injections of 10% gelatin solution (10–15 c.c./kg.). The splenic vein samples behaved as before.

(4) Two dogs were given an anaemia by a single large haemorrhage of $\frac{1}{20}$ of their body weight 4 days previously. The results were unaltered.

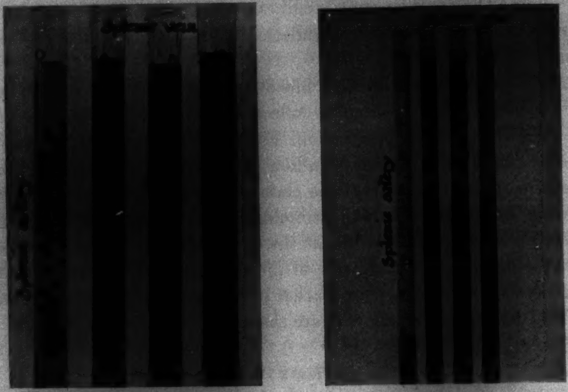
Splenic vein
Splenic artery
Splenic artery

Fig. 4*a*.

Fig. 4*b*.

Fig. 4. Resting spleen. Dog after haemorrhage. Left: 2 hr. sedimentation. Right: haematocrits of same tubes. (Nembutal.)

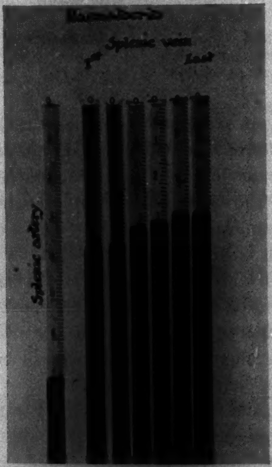
Haematocrit
Splenic vein
1st
Last
Splenic artery

Fig. 5. Resting spleen. Haematocrits of splenic artery and vein blood of dog after haemorrhage 4 days previously. (Nembutal.)

Fig. 4 shows the sedimentation and haematocrit results in one of these animals (nembutal anaesthesia). The values are shown in Table IV.

TABLE IV

	Haematocrit mm.	Sedimentation rate (1 hr.) mm.
Splenic artery	18	30
1st splenic vein sample	57	less than 0·5
2nd ,,	58	less than 0·5
3rd ,,	59	less than 0·5

The diffuse upper level of the arterial sedimenting column after haemorrhage, as shown in Fig. 4, is due to the presence in this diffuse zone of immature red cells [Stephens, 1938*b*].

Altogether twenty-four samples were taken from the splenic vein in each dog. In Fig. 5 the haematocrits over the full range of splenic vein samples of the second dog are shown.

ANAEMIA AND RED CELL CONCENTRATION BY THE SPLEEN

It is worthy of mention that the spleens of dogs apparently concentrate the blood to at least the same relative extent after haemorrhage when the red cell count of the general circulating blood is low, as when the blood count is normal. This can be seen by comparing Figs. 4 and 5 from anaemic blood with Fig. 2 from normal blood. These haematocrit values are shown in Table V.

TABLE V

	Splenic artery haematocrit mm.	Maximum splenic vein haematocrit mm.
Typical normal dog, Fig. 2	41	79·5
Anaemic dog, no. 1, Fig. 4	18	59
Anaemic dog, no. 2, Fig. 5	29·5	69

The spleens of these animals were not disturbed, and the entire splenic mesentery was clamped immediately at laparotomy under nembutal anaesthesia. All these spleens and those of other normal dogs under nembutal were observed to be extremely dilated. Their colour was dark violet, approximately the colour of reduced blood, the general appearance being the same in all cases. The actual dimensions of the spleens were surprisingly large but these are probably the dimensions of the spleen in a living normal dog at rest [Barcroft & Stephens, 1927]. The spleens could not be weighed without interfering with the experiment. The maximum thickness was between 3 and 4 cm. in each case, the other dimensions being shown in Table VI.

TABLE VI

	Body weight kg.	Size of spleen at laparotomy (cm.) Max. length cm.	Max. width cm.
Normal dog	14·5	26·5	16·5
Anaemic dog, no. 1	17·5	27	14
Anaemic dog, no. 2	22	29·5	14·5

The fact that the spleens were thus engorged with blood under conditions where a haemoglobin deficiency in the general circulation is to be presumed is perhaps surprising. There is, however, no reason to attribute the phenomena to the anaesthesia, as the result accords with the observation of Shen [1928] that anaemia does not cause contraction of the spleen. Although ether anaesthesia does not produce such marked splenic engorgement, its vasodilator action could account for the difference, vasodilators causing marked splenic contraction.

CAUSE OF SEDIMENTATION RATE DIFFERENCES BETWEEN SPLENIC ARTERIAL AND VENOUS BLOOD

The low sedimentation rate of splenic vein blood is possibly due, not only to the concentration of red cells, but partly to splenic influence upon it. It is difficult rigorously to separate the two factors by experimental means.

In three of five cats in which the principal splenic vein and several of the smaller veins were ligated, anomalies were found which would seem to be outside the limits of experimental error. The indications were that the plasma protein concentration had been increased, simultaneously with the increase in the red cell concentration. Thus the values shown in Table VII were found 70 min. after the spleen had been emptied and the veins ligated:

TABLE VII

	Sedimentation rate (90 min.) mm.	Haematocrit mm.
Splenic artery	41	22·0
1st splenic vein	43	32·5
2nd ,,	16	35·0
3rd ,,	17	33·0
4th ,,	4	41·0
5th ,,	less than 0·5	52·0

The first splenic vein sample has thus a sedimentation rate slightly greater than that of the artery, but its haematocrit value is nevertheless also greater by 50%. Cases of this sort may occur more frequently than stated above but be concealed by mixing of blood on emergence from different regions of the spleen or in the cannula. (The cat from which the data given in Table VII were obtained exhibited an anaemia and high sedimentation rate for which no cause could be assigned.)

A simple interpretation of these anomalies to mean that the plasma protein composition has been altered is not, however, possible. Alteration of red cells and of their surfaces by the spleen must be taken into account here and also in explaining the abnormally low sedimentation rate of splenic vein blood. In the latter case Bergenhem & Fåhraeus [1936] suggest the action of a lysolecithin-like substance. The question as to whether red cells of some particular type have selectively been arrested in the spleen pulp will be considered subsequently.

SEDIMENTATION FACTORS IN THROMBOSIS OF THE SPLENIC VEIN

In two cats whose blood had high sedimentation rates of 20·5 and 23·0 mm./hr. (normal value 3 mm./hr.), it was noticed that a sedimented deposit formed inside the splenic vein when the blood had lain stagnant for approximately 10 min. after the vein and arteries were clamped. This was seen in the course of experiments in which the spleen had been refilled immediately before the vessels were clamped, and the cannulation purposely delayed. In these cases the vein, approximately horizontal, was occupied above by clear plasma with a sedimented deposit of corpuscles below. Formation of red cell aggregates *in vivo* in conditions of stasis is well known and is discussed by Fåhraeus [1929]. The present phenomena represented the later stage of sedimentation after aggregate formation.

When, later, the splenic vein blood was withdrawn, the initial sample showed haematocrit values of 17 mm. in one animal and 20·5 mm. in the other, the splenic artery haematocrit values being 29 and 30 mm. respectively. Evidently the low red cell concentration and increased plasma protein content (presumed from the high sedimentation rate of the general circulating blood) were the two factors causing the rapid *in vivo* sedimentation in these cases.

In another cat in which the principal and several of the accessory veins had been ligated for some time, the blood sedimented 2–3 mm. inside the splenic vein cannula of 6 mm. bore, within approximately 3 min. between the taking of samples.

It is conceivable that the frequently occurring thrombosis of the splenic vein in the human subject arises from similar rapid sedimentation in conditions of stasis brought about by contraction of the splenic vein.

CONCENTRATION AND SEDIMENTATION RELATIONSHIPS IN DENERVATED EXTERIORIZED SPLEEN

In one dog only have these measurements been made. The nerves of the dorsal portion of a dog's spleen were cut, care being taken to ensure that the nerves entwining the blood vessels supplying this region were divided. The innervation of the ventral half of the spleen was not disturbed. The spleen was then exteriorized, the experiment thus being similar to that previously described [Barcroft & Stephens, 1927].

Blood samples were taken 15 days later by puncture of the spleen, and for this purpose no anaesthetic was necessary. It was possible to obtain samples for sedimentation measurement only from the denervated end. Clotting always occurred in blood from the other end of the spleen. It was noticed, moreover, that when the denervated end was punctured, blood flowed out quite readily, but from the end with the nerves intact a slow oozing only appeared.

The following measurements were made:

	Sedimentation rate (1 hr.) mm.	Haematocrit mm.
Femoral vein	33	29·5
Denervated spleen	18·5	32

In this experiment there was very little concentration of blood by the denervated spleen. The low sedimentation rate of the splenic blood may be considered anomalous, the haematocrit difference probably not accounting for it.

SUMMARY

1. Concentration of red cells occurs within a few minutes during filling of cats' and dogs' spleens. The cells are strained out of the blood and the plasma passes on. In cats a further concentration ensues when the principal splenic vein alone is clamped.

2. In anaemia the spleen of the dog concentrates blood to the same relative extent as normally.

3. Blood from the splenic vein of resting spleens of cats or dogs has a much lower sedimentation rate than blood from the artery.

4. Immediately after refilling the spleen the first vein sample shows a much greater sedimentation rate than the artery, sometimes four or five times as great. This is due to red cells being retained in the spleen, impoverishing the initial samples and enriching the later ones.

5. The results are unaltered when the sedimentation rate of the general circulation is abnormally high, or in anaemia.

6. Most of the sedimentation differences between splenic artery and vein may be adequately explained by the varying red cell concentration. Altered splenic vein plasma composition or red cell surface changes may also occur.

7. Ether or urethane diminish the sedimentation rate of the general circulating blood, probably due to altered plasma composition. Ether or chloroform and ether anaesthesia produce a marked increase in red cell electrophoretic speed.

8. Thrombosis of the splenic vein in the human subject may be due to the high sedimentation rate of some portions of the emergent blood, in addition to stasis caused by contraction of the vein.

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THE "CIRCULAR" MUSCULATURE OF THE SMALL INTESTINE

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In 1914 Gunn & Underhill published records of the contractions of the cat's isolated muscularis mucosae. The technique which they had to use to get good contractions suggested to them that "the muscularis mucosae of the cat's intestine consists, at least in part, of fibres which run, not in a directly circular or longitudinal direction, but obliquely or spirally". Between 1919 and 1921 Carey published the results of his studies on the arrangement of muscular and other fibres in the small intestine. As his work does not appear to be very widely known, the main results may be summarized here. The species upon which he experimented were the dog, cat, pig, sheep and cow. In all of these the inner and outer muscular layers, i.e. those layers commonly called "circular" and "longitudinal", were found in reality to be "close spiral" and "long spiral" layers respectively; they ran in a counter-clockwise direction from above downwards. The continuous "close spiral" musculature made one complete turn in 0·5–1·0 mm. or less, and was considered to be responsible for the localized wave of contraction occurring during propulsion of food material along the small intestine. The "long spiral" musculature, on the other hand, made one complete turn in every 20–50 cm. or more, and was responsible for the dilatation that preceded the contraction down the intestine. In 1932, however, Goerttler published conclusions diametrically opposed to those of Carey with regard to the musculature of the *human* intestine. It is difficult to assess Goerttler's findings, for they were very briefly stated in a paper of 50 pages. A further contribution to the subject was made in 1934 by Reid, Ivy & Quigley. These authors had introduced artificial boli of cotton and rubber into the proximal end of Thiry-Vella fistulae of the jejunum in dogs, and had found that these boli usually rotated 360° in passing through a length of 23–35 cm. of intestine, the direction of rotation being counter-clockwise from above downwards. They had also noted rotation of "gyrometers" introduced into the small intestine, and by radiographic means had shown that the rotation occurred in the one direction only.

One of the present writers, as a result of reading Carey's papers, has for some years been interested in anatomical and functional instances of spirality occurring within the body. In many veins there is a spiral arrangement of collagen fibres [Franklin, 1937, pp. 39–40], and Häusler in 1933 produced presumptive evidence of a similar disposition of the muscle fibres in mesenteric arteries. One might suppose that the trachea and bronchi would resemble the alimentary tract from which they are an outgrowth, but Miller's summary [1937] makes it clear that the musculature is arranged in a "geodesic" network rather than spirally, and a few physiological experiments made by ourselves on the tracheae of the cat and of the rabbit were also against the presence of a spiral arrangement. On the other hand, foreign substances are carried by ciliary action spirally upwards in the bronchi and trachea, the direction of the spiral being clockwise, viewed from above. Normally, inhaled dust and injected Indian ink travel up in this way [Barclay, Franklin & Macbeth, 1937; Barclay & Franklin, 1937], and radio-opaque fluids have been observed, by means of the fluorescent screen, to pass spirally up the trachea [Franklin & Janker, 1938, unpublished].

With such diverse instances of spirality occurring within the body, it seemed to us to be worth while to make some further studies upon the small intestine.

METHODS AND RESULTS

The first experiment was performed upon a cat, anaesthetized by an intraperitoneal injection of nembutal (0·75 c.c. of 5 % solution per kg.), and 1 : 2 chloroform-ether mixture. The abdomen was opened in the mid-line, and a portion of the more distal small intestine exposed. A red-headed pin was then passed through the intestine from a point directly opposite the mesenteric attachment to this attachment. A blue-headed pin was thereafter passed through, in similar fashion, about 7·5 cm. farther down the intestine. Finally, the vessels supplying the part were tied off, and the piece of gut exsected. The cat was later killed.

The exsected portion of gut was suitably weighted at its distal end and transferred to a beaker of oxygenated Ringer's solution kept at body temperature, the red-headed pin being held in a fixed position above, while the lower end of the piece of gut was free to move at will. The blue head of the distal pin soon moved round about 90° in a clockwise direction, viewed from above. Thereafter, for about 2 hr. (the total duration of the experiment), it kept twisting quickly round to about 180° and relaxing slowly back to about 90°. These movements were often independent of the up and down movements of the gut, produced by the "longitudinal" muscle.

Qualitatively, and approximately quantitatively, similar results were subsequently obtained, and that without a single exception, on preparations from other cats and also from rabbits. The only marked point of difference in the individual experiments was in the degree of initial twist of the gut before the onset of the rhythmical movements. In two cases the rapid contractions and slow relaxations were still continuing, in practically undiminished intensity, after several hours. The movements were increased in amplitude by addition of arecoline hydrochloride, and reduced by addition of adrenaline hydrochloride, in experiments in which these drugs were tried.

Finally, in a cat anaesthetized with nembutal solution, the abdomen was opened and pins passed, as above described, through two points in a length of small intestine, with the circulation continuing. The intestine was then divided above and below the proximal and distal pins respectively, and the mesenteric attachment cut along this length. The proximal end of the segment of gut, so freed, twisted in an anti-clockwise direction, viewed from above, and the distal end in a clockwise direction.

DISCUSSION

If the fibres of the outer muscular layer of the small intestine are really longitudinal, as Goerttler stated them to be in human beings, then they could only produce up and down movements of an exsected piece of gut. If, on the other hand, they are "long spiral", we have to fall back on the only figures available for the degree of spirality, i.e. the figures given by Carey. A complete revolution of his "long spirals" occurred in 20–50 cm. or more of intestine, and, on this basis, in our lengths of 7·5 cm. of exsected intestine, the "long spirals" could have shown a twist of from under 50 to 135°. It would, therefore, have been impossible to say that "long spiral" musculature was excluded as a factor in producing the twisting movements that we saw, had we not observed these movements at times when there were no up and down movements of our exsected portions of intestine, and when there were no visible contractions of the "longitudinal" musculature.

If, on this evidence, we can eliminate the "longitudinal" musculature, we may next state that we can see no way in which purely circular musculature could produce twisting movements of free-hanging segments of intestine. Hence we are forced to the conclusion that some, at least, of the muscular fibres of the small intestine, in the species studied, are arranged in a relatively close spiral, and that the direction of this spiral is counter-clockwise from above downwards. The fibres must also form a continuous layer, if twisting movements are to be caused at the free end of a portion of exsected intestine, for the twist must begin from the end that is fixed. The movements were fairly powerful, so a large number of muscle fibres must have been involved.

In his experiments Carey found that localized exsection of the "close spiral" musculature stopped the peripheral progress of localized contractions along the small intestine. This agrees with our own hypothesis about the effects of the contraction of the "close spiral" musculature that was responsible for the twisting movements of the exsected intestinal segments. For, whereas the distal ends of the segments that we used were free to rotate in the Ringer's solution, in the body there would be considerable opposition to such rotation from above and below, as is obvious from the last experiment that we have described. And, if *in situ* the two ends of any portion of intestine are prevented from twisting to any great extent, the effect of the contraction of a close spiral layer of muscle should be a partial or complete diminution of the gut lumen.

Finally, though our experiments seem to favour Carey's view that there is a "close spiral" layer in the small intestine, they do not, of course, disprove the presence of purely circular muscle in this portion of the alimentary canal.

SUMMARY

Experimental evidence is given, which supports Carey's view that there is a close spiral arrangement of muscular fibres in the small intestine of certain species. The direction of the spiral is anti-clockwise, viewed from above (stomach end of the intestine). The physiological implications are discussed.

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ON THE MODE OF ACTION OF VISUAL PURPLE ON THE ROD CELL

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This paper deals with some unexpected results obtained in parallel measurements of visual purple concentration and size of the dominant initial *b*-wave of the retinal electrical response. Some general correlations between the properties of visual purple and the *b*-wave have already been established. Thus we know that the size of the *b*-wave reproduces the absorption curve for visual purple (see, for example, a summary by Granit [1938]). But more important for our purpose than this static criterion is a dynamic one, based on the work of Tansley [1931] and Charpentier [1936]. The former measured the concentration of visual purple, the latter the size of *b*-wave of the retinal electrical response during dark adaptation of white rats previously adapted to light. It was found that the rise in concentration of visual purple during regeneration was accompanied by an equivalent rise in size of the *b*-wave. The same conclusion is also emphasized by the work of Wrede [1937] and Riggs [1937]. It would seem therefore that the size of *b*-wave, at least during regeneration of visual purple, might be directly determined by the total amount of visual purple available.

So far, however, no strictly parallel measurements of the retinal electrical response and the density of visual purple extracts have ever been made in any laboratory, and, though the results referred to are very definitely established, it is not known, for instance, whether a reduction of the electrical response due to moderate light adaptation is accompanied by a parallel reduction in the concentration of visual purple. For this purpose it was found necessary to develop a technique for measuring the concentration of visual purple from single eyes (see below). Having done so we soon found that adaptation to different monochromatic lights which caused a considerable reduction in size of the *b*-wave, elicited by a test light, did not lead to a measurable reduction of the amount of visual purple obtainable from the eye used. The evidence for this conclusion is given below.

As we have pointed out in a preliminary note [1938], this lack of correspondence during moderate light adaptation between the behaviour of the *b*-wave and the amount of visual purple during regeneration is of great theoretical interest. It is important to know how accurately visual purple of individual eyes can be determined. Below we give details of our technique and procedure.

TECHNIQUE AND PROCEDURE

Photoelectric technique. The requirements of the technique for measuring V.P. (visual purple) densities are: (i) low intensity of the light reaching the cell in order to avoid bleaching the photosensitive substance; (ii) reasonably monochromatic light adjusted to match the highest point of the V.P. absorption curve around 0·500μ.; (iii) enough sensitivity to enable accurate working with about 0·4 c.c. of V.P. extract. These requirements were fulfilled by the apparatus illustrated diagrammatically in Fig. 1.

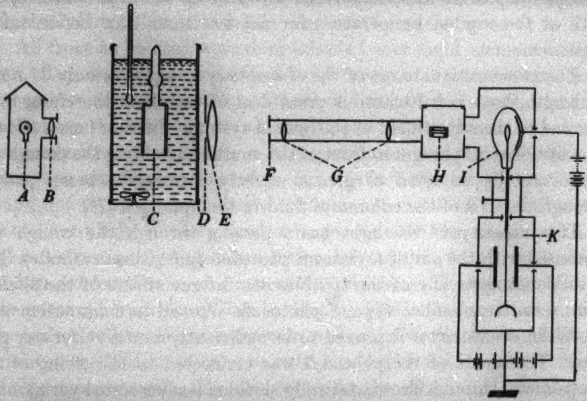

Fig. 1. Diagram of apparatus for photoelectric absorption measurements. *A*, lamp; *B*, condenser lens (10 cm. focal length); *C*, Christiansen dispersion filter in water bath fitted with electric thermo-regulator; *D*, diaphragm; *E*, lens of focal length 75 cm.; *F*, diaphragm; *G*, lenses required by optic cell; *H*, light-tight case for optic cell; *I*, photocell in dried case; *K*, string electrometer with connexions. Not to scale.

The light source was a small bulb of the automobile headlight type run off a storage battery below its rated voltage. This light was passed through the Christiansen dispersion filter (Schott and Gen. Jena). This filter contains minute particles of glass in an organic fluid (an ester of benzoic acid) and has to be used in conjunction with the diaphragm (*F*) and lens (*E*) forming an image of the filament on the adjustable aperture of the diaphragm. For each temperature of the bath containing the filter the refractive indices of the glass and the fluid inside the filter are identical only for a narrow spectral region. In the system used, therefore, the image on the aperture of the diaphragm is monochromatic. In our experiments the water bath was kept at 33·00° C. with the aid of a mercury thermoregulator. Spectroscopic tests showed that the maximal intensity then fell at 0·498μ. With a diameter of 35 mm. of lens *E* and an aperture of 4 mm. of the diaphragm the total spectral range covered was 0·008μ. It proved to be important to warm up the filter and keep the bath at the wanted temperature for not less than 1 hr. before taking measurements.

The cross-sectional area of the absorption trough was only 17 mm.^{2}, its length, however, 20 mm. A great deal of care had, therefore, to be devoted to the adjustment of the optical system. Further lenses (*G*) and an additional diaphragm in front of the container holding the trough (*H*) were carefully adjusted to give a slightly convergent beam passing through the axis of the column of fluid in the optic cell (*H*).

The intensity of the light beam passing through the trough was measured with the aid of a vacuum photocell (potassium cathode). This cell did not show any of the troublesome fatigue effects of the slightly more sensitive gas-filled type of photocell. Worked in conjunction with the Wulff electrometer it proved to be sufficiently sensitive for our purposes. The anode of the photocell was connected to the string of the electrometer through electrostatically shielded leads isolated with amber, the surrounds being kept dry by means of phosphorus pentoxide. This degree of insulation proved sufficient to enable us to measure the photoelectric current by observing with a stopwatch the time necessary for the development of a given charge corresponding to a deflexion of 20 points on the scale in the eyepiece of the electrometer. The value obtained is inversely proportional to the intensity. Controls were made with six carmine solutions, diluted in the ratios of 1 to 1/2, 1/4, 1/8, 1/16, and 1/32. Single measurements gave the ratio of their densities as 2·01 instead of the theoretical value 2·00. In the experiments the same solutions were often repeatedly measured. The maximum difference from the mean value amounted to about 1%, but quite often successive readings gave identical values. Some practice with the stopwatch guaranteed an accuracy of about 0·5%. It was held unnecessary to develop a better method of timing.

The visual purple extracts. The important point was to obtain *all* the visual purple from a given retina. A great deal of attention has recently been devoted [Lythgoe, 1937; Chase & Haig, 1938] to purification of the extracts in order to obtain the true absorption curve for visual purple, but such methods are too elaborate for our purpose owing to the greater risk of unevenly distributed losses in the different stages of purification. The presence of impurities is of little importance when the amount of V.P. has to be measured as a difference between the readings for an unbleached and a completely bleached solution at a single wave-length.

The method chosen for extraction was the one used by Tansley [1931], but it was improved upon by paying more attention to centrifugation. We shall show below that it was adequate for our purpose.

All frogs to be used were dark-adapted over night at room temperature. Removal of the retinae had to be carried out with extreme care as the success of this operation, other conditions being equal, ultimately determines the accuracy of our conclusions. If the retinae do not come out easily too low values for V.P. concentration are obtained. At times some pigment clings to the retina, but this fact, against expectation, was found not to influence the measurements. Removal of the retinae took place in red light of wave-lengths longer than $0{\cdot}615\,\mu$. Each retina was put in 1 c.c. of a 2% digitonin solution, left standing for 40 min. and then centrifuged for 20 min. at a rate of 3800 rev./min. The digitonin solution was prepared fresh every day by boiling. The centrifuged V.P. extracts are perfectly clear. When extraction was complete (see below) the retinal debris at the bottom of the test-tube has lost its red colour.

The measurements. The absorption in the solution of visual purple could be measured photoelectrically without any bleaching taking place during this procedure. Thus, in one experiment the initial density was 0·442; after 17 min. of continuous exposure to the monochromatic light it was 0·437, and finally, after 34 min., 0·436. Density is defined as $D = \log_{10} I_0/I_t$, where I_0 is the intensity of the light incident on the solution, I_t the light transmitted to the recording instrument. The amount of visual purple is given as the difference between the density of the solution immediately after extraction and its density after bleaching of the visual purple for 5 min. with a 1000 W. lamp at a distance of 40 cm.

The electrophysiological technique and the bleaching of the eyes. The electrophysiological material on which the comparison is based consists of seventy experiments performed with twenty-four opened, excised and dark-adapted eyes of Hungarian frogs. The electrical response of these eyes to a constant test flash (1 sec.) of wave-length 0·500 μ. was measured with the aid of a directly coupled amplifier and a cathode ray before and after 5 min. of adaptation to certain wave-lengths of a Tutton monochromator. The results obtained have been evaluated elsewhere from other points of view [Granit, Therman & Wrede, 1938]. Here we shall use certain averages and compare them with v.p. absorption measurements from experiments which were carried out in exactly the same manner up till the moment when, after bleaching, the retina was put into digitonin instead of being tested for its electrical response. A very brief description of the technique of adaptation to monochromatic light should therefore suffice, a detailed account being found in the paper referred to.

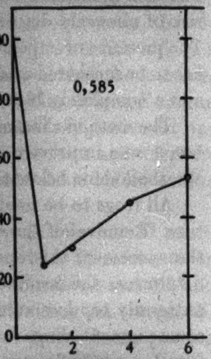

Fig. 2. Bleaching *in vivo* (or adaptation) to wave-length 0·585 μ. Ordinates: relative amounts of visual purple calculated from the size of the *b*-wave of the electrical response. Zero abscissa shows amount of visual purple before adaptation. Abscissae from 1 to 6 min. refer to time after cessation of adaptation.

Initially a number of electrical responses to wave-length 0·500 μ. are measured with the dark-adapted eye. Size of *b*-wave is then of the order of 0·7 mV., but the initial values are averaged and reduced to the arbitrary unit of 100. All effects of adaptation are then conveniently given as percentage reduction of the response to the test light. The initial value having been fixed, a filter of 1·3 log units is removed from the monochromator and the eye exposed to some wave-length of the equal energy spectrum for 5 min. Then the filter is reinserted and the response to the test light during recovery measured at intervals of 1 and, later, 2 min. A typical recovery curve is illustrated in Fig. 2. Generally the eyes were exposed a second time to the adapting light for a new period of 5 min. In fact, all the eyes used for v.p. extraction were bleached twice for two periods of 5 min.

Instead of testing for size of *b*-wave with wave-length 0·500 μ. we now removed the retina after the adaptation and placed it in the digitonin solution. The operation lasted on an average 75 sec. From the curves of Granit *et al.* [1938] the amount of reduction of the retinal response at this moment could be ascertained.

RESULTS

Effect of bleaching as measured electrophysiologically. Table I shows the effect of adaptation to different wave-lengths in terms of concentration of visual purple as calculated from the size of the *b*-wave elicited by the test light 75 sec. after the period of bleaching. The figures indicate percentage reduction from the original level of 100 before adaptation. V.P. concentration is calculated on the basis of the equation of Chaffee & Hampson [1924] [see Granit *et al.* 1938]. The table illustrates the surprising discovery made by the latter authors that the effect of bleaching is not proportional to the absorption curve for visual purple; the long wave-lengths cause a much greater depression of the response to test light 0·500 μ. than the short wave-lengths. The actual amount of bleaching produced by the bleaching light is, however, very small. If the absorption trough containing the V.P. solution is placed in the beam of this light (0·500 μ) for 10 min. the effect on the absorption measured by the photo-electric colorimeter does not exceed 2–3 %. This indicates extreme sensitivity of the dark-adapted eye to light.

TABLE I. The table illustrates reduction in concentration of visual purple 75 sec. after cessation of adaptation, as it should be if calculated from the reduction in size of the *b*-wave of the electrical response. The wave-lengths of the adapting lights used are given in the upper horizontal row

Wave-lengths in μ.	0·585	0·560	0·540	0·500	0·470	0·450	0·430
Percentage reduction	74	69	77	59	56	35	27

Comparison of visual purple concentration in right and left eyes. Assuming the two eyes of the same frog to contain identical amounts of visual purple, the first experiments carried out were to determine the accuracy of our method by comparing measurements of visual purple densities in right and left eyes. The outcome of this experiment is shown in Table II for fourteen frogs. Despite variations from animal to animal the final averages for this number of frogs are identical to within 1 %. The first eight frogs were from an earlier delivery. Their averages were respectively 0·401 and 0·413 and thus differed by 3 %. The six remaining frogs (later obtained from Hungary) had eyes containing more visual purple, for right and left eye respectively 0·564 and 0·538, the difference being 4 %. Thus it can be seen that with a sufficient number of experimental animals right and left eyes contain amounts of visual purple identical to within 1% and that an accuracy of 4–6% can be obtained with averages from only four to six eyes.

TABLE II. Comparison of visual purple content in right and left eyes of fourteen frogs

Frog no.	Density in one eye	Density in the other eye	Difference %
I	0·216	0·236	+ 9
II	0·409	0·405	− 1
III	0·484	0·524	+ 8
IV	0·574	0·518	−10
V	0·429	0·486	+ 8
VI	0·403	0·387	− 4
VII	0·315	0·332	+ 5
VIII	0·379	0·416	+ 9
IX	0·709	0·687	− 3
X	0·538	0·526	− 2
XI	0·480	0·509	+ 6
XII	0·570	0·538	− 5
XIII	0·541	0·426	−21
XIV	0·545	0·541	− 1
Averages	0·471	0·467	1

Concentration of visual purple in bleached eyes. In the first set of experiments one eye of a frog served as dark-adapted control, the other eye was bleached with one of the wave-lengths shown in Table III.

TABLE III. Bleaching of one eye with different monochromatic lights, the other eye serving as fully dark-adapted control

	Control	0·430 μ.	Control	0·450 μ.	Control	0·500 μ.	Control	0·585 μ.
	0·522	0·440	0·550	0·526	0·592	0·560	0·530	0·458
	0·531	0·530	0·628	0·668	0·345	0·335	0·481	0·509
	0·454	0·502	0·442	0·492	0·429	0·445	0·330	0·366
	0·542	0·492			0·254	0·319	0·415	0·314
Averages	0·512	0·491	0·540	0·562	0·405	0·415	0·439	0·412

The table shows that for the four wave-lengths used the densities of visual purple of the bleached eyes differ from the values of the controls by −4, +4, +2, and −6% respectively. Averaging separately all the fifteen dark-adapted controls and the fifteen bleached eyes, irrespective of wave-length used for adaptation, the average density of the controls is 0·470, that of the bleached eyes 0·464. The values are thus identical to within 1%, as in Table II. Thus adaptation to monochromatic light has led to no reduction whatsoever of the concentration of visual purple measured in the photoelectric colorimeter with a light of wave-length 0·498 μ. Nevertheless, the electrical response to a test light (0·500 μ.) of practically the same wave-length flashed on to the living retina has been greatly reduced by adaptation, as shown by Table I.

Wave-lengths 0·430 and 0·585 μ. cause approximately equal responses with the low intensity rod spectrum [Granit & Munsterhjelm, 1937].

Actually the response to 0·585μ. is larger at the higher level of intensity used in these experiments, but this is due to cones taking part in the reaction in the long wave-lengths [Granit & Wrede, 1937]. From the point of view of V.P. absorption the presence or absence of active cones should be immaterial. On the other hand, the bleaching experiments of Table I have shown that V.P. concentration calculated from the reduction of the *b*-wave (for test light 0·500μ.) is 74 % for wave-length 0·585μ. as adapting light and only 27 % for wave-length 0·430μ. as adapting light. In view of this result it was decided to perform a series of experiments in which one eye would be exposed to wave-length 0·585μ., the other eye to 0·430μ.

TABLE IV. Bleaching of one eye with wave-length 0·430, of the other eye with 0·585μ.

0·430μ.	0·585μ.		0·430μ.	0·585μ.
0·432	0·416		0·439	0·506
0·342	0·412		0·416	0·414
0·483	0·548		0·452	0·397
0·437	0·545		0·417	0·364
0·331	0·504		0·316	0·452
0·359	0·263		0·530	0·486
0·388	0·367	Averages	0·411	0·436

The outcome of this experiment is shown in Table IV. The difference between the average densities is 0·025 or 6%. It is too small to be regarded as significant, though greater than the previous series would have led one to expect.

DISCUSSION

The facts presented are simple and clear cut. On the one hand earlier work has shown that the size of the *b*-wave elicited by a constant test light increases during regeneration of visual purple, on the other hand we have found above that a considerable reduction in size of the electrical response caused by adaptation to monochromatic light does not coincide with a measurable diminution of the total quantity of visual purple. In a way our result is disappointing because it means that a number of electrically measurable changes must now be regarded as being beyond reach of direct photochemical control, among them the interesting fact that bleaching with long wave-lengths reduces the response to test light 0·500μ. far more than bleaching with short ones. Nevertheless, it is a positive and significant result that unmeasurably small changes in the total concentration of visual purple are engaged in excitatory processes not far from the upper limit of maximal rod capacity.

How then are we to explain that the size of the electrical response of the retina in one type of experiment—regeneration—is some simple function of V.P. concentration, in another type of experiment—our own—does not correlate in the least with density measurements of visual purple? Our own choice of a working hypothesis is contained in the assumption that most of the visual purple is a store which although it is bleachable is nevertheless physiologically inactive and incapable of initiating nerve impulses. The concentration of the inactive visual purple must be high because by simple diffusion or otherwise it determines how much photosensitive material is available at the locus where excitation takes place. Thus we have accounted for the necessity of a high concentration of visual purple for large electrical responses, as well as for the absence of measurable photochemical changes accompanying the reduction of the *b*-wave after adaptation to moderately strong monochromatic stimuli. In the latter case only the decomposition in the relatively small fraction directly concerned in the process of excitation is physiologically significant.

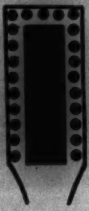

Fig. 3. The diagram illustrates the outer limb of the rod containing a store of inactive visual purple inside the cell and a layer of active stimulating photosensitive material (filled circles) along the surface.

This scheme is pictured by the diagram of Fig. 3 illustrating the outer limb of the rod cell. We have assumed that the active visual purple is active because of its particular manner of distribution, say, at the surface of the cell. The surface charge may be kept up, as suggested, by diffusion from the store of active material inside the cell. Illumination may be assumed to lead to a depolarization of the surface spreading electrotonically and giving rise to the *b*-wave of the electrical response of the retina. In this scheme excitation takes place along the whole surface of the rod cell, but this particular assumption is by no means essential. It is quite possible that excitation is initiated at some other point of still smaller dimensions, and that V.P. has to be bleached in its neighbourhood in order to excite. In fact, such a state of affairs is indicated by the presence of the "directional sensitivity" of the retina, discovered for cone vision by Stiles & Crawford [1933, 1937].

The idea of a store of photosensitive material from which the active material at the locus initiating excitation is replenished owes a great deal of its attractiveness to its simplicity combined with the many perspectives it suggests both with regard to theory and further experimental progress. Thus there is the well-known phenomenon of a heavy outburst of impulses associated with the *b*-wave which appears as an initial "explosion" leaving "refractoriness" in its wake. There is also the "silent period" following the initial discharge [Hartline & Graham, 1932; Hartline, 1938], both of which suggest the hypothesis just outlined.[1] It is also worth while pointing out that on this view the time constant of the process of regeneration of visual purple may be of relatively little immediate significance for the actual process of excitation. The "rate of diffusion" to the locus of excitation may by comparison be more important. It is also of interest for the general problem of cone vision to see that exceedingly small quantities of a photosensitive substance suffice for excitation of a visual cell.

Finally we cannot avoid mentioning a fundamentally different line of thought capable of explaining our results. This is the possibility that the visual purple of the living rod may exist in some relatively stable form comparable to its state in the "artificial retinae" which Weigert & Nakashima [1929, 1930] made by dissolving visual purple in gelatine. If this were so—a possibility by no means excluded—our results would necessitate a still more radical revision of current concepts with regard to how visual purple mediates sensations.

SUMMARY

Two methods have been used in parallel: (i) measurements of the size of the electrical retinal response with a cathode-ray oscillograph and a directly coupled amplifier, (ii) measurements of the total quantity of visual purple of single eyes (digitonin extracts) with a photocell and a Christiansen filter adjusted for wave-length $0{\cdot}498\mu$. The preparation was the dark-adapted excised eye of the Hungarian frog.

The aim of the work was to find out whether a reduction of the electrical response to a constant test light (of wave-length $0{\cdot}500\mu$.), caused by means of adaptation to monochromatic light, is accompanied by a measurable reduction of the amount of visual purple obtainable from such eyes.

Initially it is shown that the total quantities of visual purple from right and left eye of the same frog are identical to within 1% (averages of fourteen eyes). Thus one eye may be used as dark-adapted control when the other eye is illuminated.

The experiments demonstrate that the total quantity of visual purple obtained from eyes in which the retinal electrical response to wave-length

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[1] Dr H. K. Hartline in a discussion with one of us drew attention to the applicability of this idea as a possible explanation of the "silent period".

$0{\cdot}500\mu$. has been reduced by one-third to one-half by adaptation is the same as in the control eyes. The rods may therefore be forced to nearly maximal activity without incurring measurable losses in their content of visual purple.

Since, on the other hand, the retinal electrical response increases during regeneration of visual purple and thus somehow is determined by the total amount of photosensitive material available, it has been necessary to suggest a hypothesis to account for the apparent discrepancy between these two sets of results.

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NERVOUS DISCHARGES FROM THE OLFACTORY ORGANS OF FISH

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We are still extremely ignorant of the way in which sensations of smell are produced, but recently two lines of enquiry have given promising results. Elsberg and his colleagues [1935] have introduced a new method for applying measured olfactory stimuli to man and have used it in clinical investigations, and Hasama [1934] has made string galvanometer records from the olfactory parts of the rabbit's brain and shown that they become electrically negative when a smell reaches the nose. In the present work we have tried to make a more detailed analysis by electro-physiological methods. One way of securing further information would be to separate the fibres in the olfactory nerve and record the impulses in them; but attempts to do this have failed hitherto because the fibres are non-medullated and too delicate to survive the dissection. There is, however, a much greater chance of recording the olfactory discharges after they have been relayed through the synaptic region of the olfactory bulb. In certain fish the bulb is connected with the forebrain by a slender "olfactory stalk" no thicker than a small nerve trunk and composed of medullated fibres. Impulses passing up the fibres of the stalk give measurable action potentials, and we have recorded the action potentials set up by various stimuli applied to the olfactory organ.

The stalk resembles the optic nerve in that its fibres are not directly connected with the receptor cells of the sense organ, but the impulses in it, like those of the optic nerve, should be a reasonably faithful index of the sensory excitation. The connexion with the receptors is in fact closer in the olfactory system than in the optic, for the mitral cells from which the olfactory stalk fibres arise are separated by only one synapse from the receptors in the nose. Records from the olfactory stalk may therefore be expected to give fresh data on the physiology of smell, though they will not show the impulses which arise directly from the receptors of the olfactory organ.

METHOD

Material. The long olfactory stalk is found in many fresh water fish, including the carp, tench, goldfish and other Cyprinoids and the catfish (*Amiurus*). All these have been examined, but the majority of the records have been made from the catfish, partly because its tissues survive better and partly because Parker [1910] and Olmsted [1918] have demonstrated the existence of true olfactory reactions in the catfish and have tested the substances which arouse them.

The diagram in Pl. I, fig. 1, shows the general form of the catfish's brain with the olfactory stalks on either side running forwards to the olfactory bulb and the optic nerves running more laterally to the retina. The diagram is meant to illustrate the general likeness in the nervous apparatus for smell and sight, but the relative sizes of the sense organs and the paths to the brain have not been exaggerated. With some modifications in the form and size of the various lobes of the brain the diagram would apply equally to the carp and tench.

The olfactory organ consists of a number of thin plates projecting into the cavity of the olfactory sac so as to present a large surface to the water flowing through it. The plates are covered with ciliated epithelium interrupted by mucous cells and slender olfactory receptor cells (Pl. I, fig. 2). From the latter non-medullated nerve fibres pass back to the olfactory bulb which lies immediately below and behind the organ. This arrangement is common to all the fish under consideration.

The olfactory organ is contained in a sac which is open to the water through a short tube or nipple which projects forwards from the skin over the front of the organ (Fig. 3) and through a slit or crescentic opening in the skin over the caudal end. The arrangement of these accessory structures varies somewhat in the different fish, the nipple being much smaller in the catfish than in the tench. The flow of water through the sac is partly due to ciliary action and partly to the water currents round the head caused by the action of the gills and by swimming movements.

The olfactory stalk in the catfish may be as long as 2 cm. (in a fish measuring 16 cm.). Sections stained with osmic acid show it to be made up of medullated fibres, mostly of 3–4μ. diameter. On a rough estimate there are 500–1000 of these fibres in the stalk. Near the forebrain the stalk divides into a thicker medial and a thinner lateral strand. From the

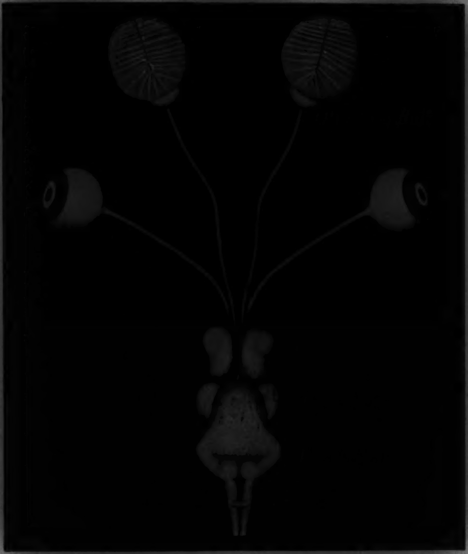

Fig. 1. Brain of catfish (*Amiurus*) showing olfactory organs and long olfactory stalks.

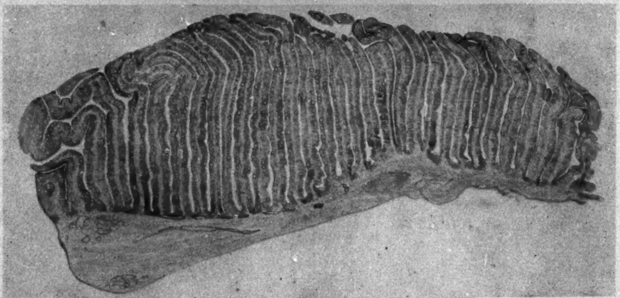

Fig. 2 A. Section through the olfactory organ showing the arrangement of the plates.

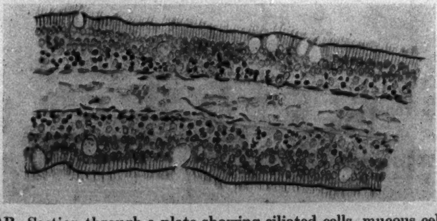

Fig. 2 B. Section through a plate showing ciliated cells, mucous cells, etc.

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account given by Sheldon [1912] it appears that the centripetal fibres arise from mitral cells in the olfactory bulb, as in mammals. The arrangement is given in the diagram in Fig. 4. Each mitral cell is in touch, through the glomerular synapses, with several olfactory nerve fibres. Smaller cells without long axons are also present in the bulb, and in the stalk there are centrifugal fibres from cells in the end-brain. Thus the olfactory pathway is reminiscent of the optic pathway but has only one synapse between receptor and brain instead of two.

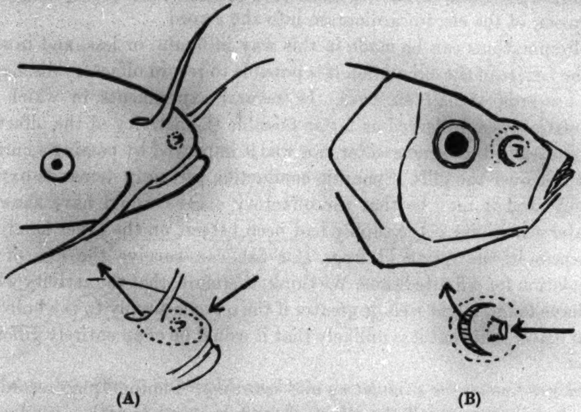

Fig. 3. External view of the olfactory organ in (A) catfish and (B) tench.

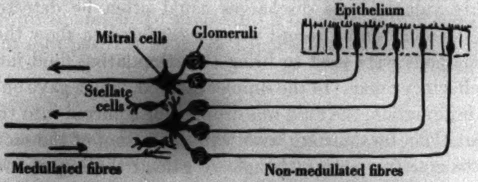

Fig. 4. Arrangement of neurones in the olfactory bulb.

Preparation. Most of our observations have been made on preparations deprived of circulating blood. The fish is decapitated and the neuraxis cut through and destroyed behind the forebrain lobes. The head is then pinned to a cork plate and the lobes of the forebrain are separated and dissected free with the attached olfactory stalks. The stalk can then be subdivided with fine needles. Finally, the forebrain lobe is lifted on to a small ring of silver wire which forms one electrode, so that the stalk hangs in the air between the forebrain and the cranial cavity. The other electrode (silver wire) makes contact either with the stalk or with some part of the head. Both electrodes are coated with silver chloride to diminish polarization effects, and the heart is removed to prevent the intrusion of the electrocardiogram into the record.

Preparations can be made in this way in 5 min. or less, and in spite of the failure of the circulation it is possible to record olfactory discharges for upwards of half an hour. In several experiments in which the circulation was preserved as far as possible the activity of the olfactory system seemed to be no greater, nor was it improved by passing a current of water over the gills. Synaptic conduction certainly demands oxygen supply, and it may be that the olfactory organs would have shown a greater sensitivity if the supply had been better; on the other hand, the synapses in the retina of frogs and fish can survive the loss of the circulation for a limited time. We think, therefore, that the activity which we have found might well be greater if the oxygen supply to the bulb had been better, but that it is unlikely that it would be of an entirely different order.

Arrangements for stimulating and recording. Photographic records of the electric response of the olfactory stalk were made with a condenser-coupled amplifier and Matthews' oscillograph, but a loud-speaker was particularly valuable and discharges could often be detected by ear although the oscillograph record was too confused to show them.

Two methods were used to bring the stimulating fluid into contact with the olfactory organ. In the simpler method, which gave on the whole the more information, the fluid was dropped or squirted into the sac and washed out again by squirting with water. The roof of the sac was often removed to give free access, though we gained the impression that the organ survived better if the sac was left undamaged and the fluids were introduced through the oral or aboral opening. Injecting the fluid in this way provides for a rapid rise in the concentration of the stimulating substance in the sac and is therefore capable of giving an intense olfactory stimulus. It has the disadvantage of causing some mechanical disturbance in the olfactory organ, and it is often difficult to avoid a displacement of the recording system owing to capacitative effects when the fluid is dropped into the sac.

The second or "continuous flow" method gives less disturbance and a less abrupt stimulus. Water is allowed to flow slowly through the sac from a glass cannula in the oral or aboral opening, and the stimulating fluid is then made to take the place of the water. The arrangement is shown in Fig. 5. The cannula has a vertical side tube which can be filled with the stimulating fluid. The taps, T_1–T_2, control the flow from the wash bottle and the side tube, and it is not difficult to close T_1 and open T_2 in such a way that there is very little alteration in the rate of flow through the sac when the change is made. The cannula must be fixed so that its orifice is in one of the openings into the sac and cannot shift when the taps are manipulated. To ensure this it is held rigidly in a heavy stand and brought into position by screw adjustments.

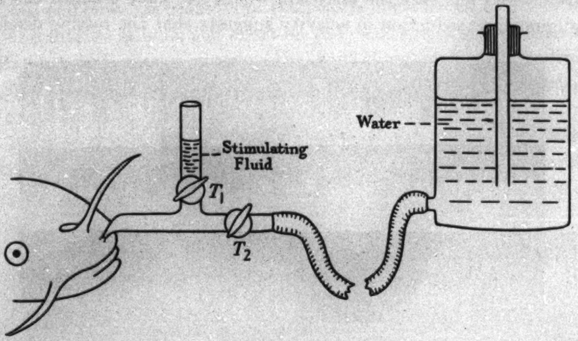

Fig. 5. Arrangement for chemical stimulation by the "Continuous flow" method.

Results

Resting discharge. During the period of survival of the olfactory system small potential oscillations are constantly occurring in the stalk, although nothing but tap water or distilled water has had access to the sac. In preparations in which the stalk has been cut down to a narrow strand of fibres it can be seen that this electrical activity is due to the repeated passage of action potentials travelling mainly in the direction of the forebrain. As a rule the frequency in the individual fibres seems to be very low and many of them may be inactive; but if the stalk is cut through near the bulb the sudden disappearance of the activity leaves no doubt of its previous existence.

Fig. 6 gives three records from a catfish preparation in which only the lateral division of the stalk was preserved. The preparation was in very good condition (judged by the response to stimulation) and the resting discharge was larger than usual. The first record shows the discharge with the sac containing tap water. The second shows the effect of placing a crystal of novocain in the sac, and the third that of cutting the stalk just behind the bulb. The second record was made within a few moments of adding the novocain, in the hope that the olfactory receptor cells would be anaesthetized but that the novocain would not have reached the bulb. The immediate reduction in activity suggests that the resting discharge is mainly due to the receptors; but as there is a further drop in activity when the stalk is cut, the cells of the bulb may continue to discharge occasionally after the receptors have been anaesthetized.

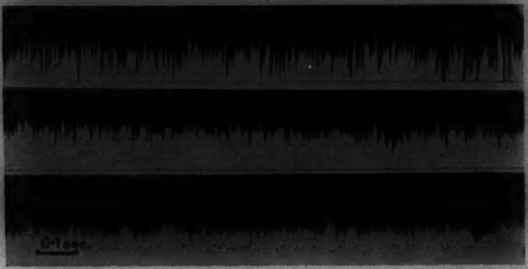

Fig. 6. Resting discharge. Olfactory stalk of catfish. A thin strand from the lateral division of the stalk is on the electrodes and the recording system is working at high sensitivity. A. Organ recently washed with water. B. Novocain in olfactory sac. C. Olfactory stalk cut through behind the bulb. Time in this and all records is given by the black horizontal line.

The olfactory receptors are not peculiar in giving a persistent discharge at a low frequency in the absence of definite stimulation. Most varieties of slowly adapting receptor give similar "resting discharges", and it is sometimes impossible to reduce them to inactivity except for a short period following stimulation. It will be seen later (p. 452) that the olfactory organ may show a similar inactive period after stimulation, the resting discharge returning gradually to its former level. Apart from these effects the resting activity shows little variation from moment to moment: there is, however, a progressive decline which runs parallel with the decline in the response to chemical stimulation, the organ becoming completely inactive after a period varying from half an hour to an hour and a half. The failure seems to be accelerated by increased activity, for an organ which has been stimulated repeatedly may fail within half an hour, whilst its fellow, which has not been touched during that period, can still give a vigorous response.

It may be mentioned here that the changes in the level of activity produced by stimulation, as well as the gradual failure of the organ, are not correlated with any marked change in the beat of the cilia which cover the olfactory plates. The ciliated cells remain active long after all nervous response has ceased and the beat is not affected by fluids which have an intense stimulating effect on the receptors. On the other hand, the amount of mucus covering the epithelium may well modify the sensitivity of the organ and an early failure can sometimes be traced to increased secretion from the mucous cells.

Mechanical stimulation. The simplest way of producing a discharge of impulses in the olfactory stalk is to stimulate the organ mechanically. Pressure on the roof of the sac is usually effective if it is enough to cause some deformation of the organ: if the sac has been opened, a discharge can be produced by touching with a brush or with moist filter paper. Repeated discharges can be set up in this way without any obvious reduction in activity; nevertheless, the excitation is most probably due to injury, for the stimuli which are most effective could scarcely avoid damaging the delicate structure of the olfactory plates, and in normal life it is unlikely that the organ would ever be exposed to them.

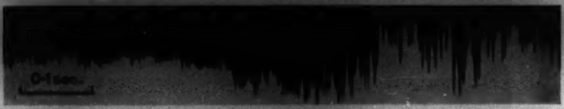

Fig. 7. Effect of mechanical stimulation. Record from entire olfactory stalk. Organ stimulated by pressure on the roof of the sac.

The essential stimulus seems to be a mechanical disturbance of the plates rather than of the olfactory bulb, for pressure is just as effective at the oral end of the sac, where it would avoid the bulb, as at the aboral. The record in Fig. 7 shows the intense discharge which may be produced by firm pressure.

As the olfactory organ is sensitive to mechanical stimuli, it is sometimes difficult to decide whether a fluid stimulates in virtue of its chemical effect or of its containing small particles which impinge on the plates when the fluid enters the sac. Small particles can certainly act as a mechanical stimulus, for water containing emery powder in suspension will give a discharge if it is injected rapidly. It is found, however, that a discharge started in this way persists only as long as there is a flow of the fluid through the sac. This makes it possible in most cases to distinguish between mechanical and chemical stimulation, for with the latter the continued movement of the fluid is not essential.

Chemical stimulation. Water containing decomposing animal matter acts as a powerful stimulus to the olfactory apparatus, and in most of our observations on chemical stimulation we have used fluids of this kind. The two which gave the most consistent results were made by allowing (*a*) earthworms and (*b*) the head of a small alligator to rot in 25 c.c. of water. Both fluids had a foul smell and were slightly turbid from bacteria and small particles of decayed tissue. They were less effective if the solid particles were filtered off, but their stimulating effect was certainly due to their chemical and not to their mechanical properties, for the discharge would persist for 10 sec. or longer if the fluid remained in the sac and would cease at once when the sac was washed out with water. This implies something more than a mechanical stimulation, for a suspension of some other kind of particle, e.g. of brain tissue fixed in formalin and ground to a paste, will give no more than a brief discharge at the moment when it is injected.

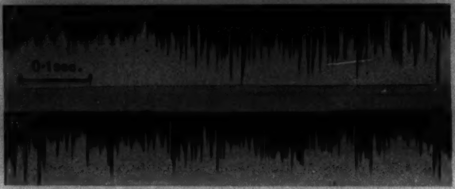

Fig. 8. Effect of chemical stimulation Olfactory organ stimulated by addition of a drop of fluid from decayed animal tissues (alligator). A shows the beginning of the discharge 0·6 sec. after the fluid was added. B shows the discharge 7 sec. later, the fluid remaining in the sac.

Fig. 8 shows two sections of a continuous record of the discharge produced by chemical stimulation. The olfactory sac has been opened by removing the roof, and the olfactory stalk has been subdivided to reduce the number of fibres in action. The first part of the record shows the occasional impulses of the resting discharge followed by the great increase in activity due to a drop of the stimulating fluid (decayed alligator). The moment of stimulation does not appear in the record, for there is a considerable latency, and in this case the drop was placed in the sac 0·6 sec. before the discharge began. In the second part of the record taken

7 sec. later, the discharge still continues though with a diminished frequency. Eventually, 10 sec. after the start, the sac was washed out with water and the discharge ceased.

Evolution of the discharge. A response of this kind might have been produced by any of the fluids which we have tested, for none of them has given a characteristic frequency or grouping of impulses, though there have been minor differences in the evolution of the discharge. Differences in the form or frequency of the impulses might, perhaps, become apparent in records made from single fibres of the olfactory stalk, but we have not yet succeeded in making preparations with so few fibres that the impulses in each could be clearly distinguished. For this reason we cannot give an accurate estimate of the frequency of the discharge from moment to moment. An occasional series of impulses can sometimes be made out in preparations in which the stalk has been cut down to a slender filament. In Fig. 9, for instance, it is possible to distinguish a fairly regular sequence at a frequency of about 40 a sec., and in Fig. 10 the initial increase in frequency can be followed. By counting every excursion above a certain size the rise and fall in the intensity of the discharge can be very roughly estimated, and Fig. 11 has been made in this way from the record illustrated in Fig. 8.

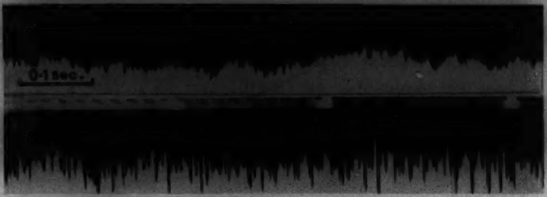

Fig. 9. Chemical stimulation by the continuous flow method. Record from small filament of the olfactory stalk. The stimulating fluid is water from fresh earthworms. A before and B during the passage of the stimulating fluid through the sac.

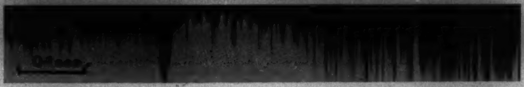

Fig. 10. Chemical stimulation by drop method. Record from small filament of olfactory stalk. The fluid is water from decayed earthworms. The large downward excursion before the discharge is due to the forebrain and not to the olfactory system.

From this and similar observations it is clear that the olfactory apparatus (the organ and the bulb) reacts with a certain inertia and has a rate of adaptation to the stimulus which cannot be classed either as very slow or as very rapid. Even with an intense stimulus

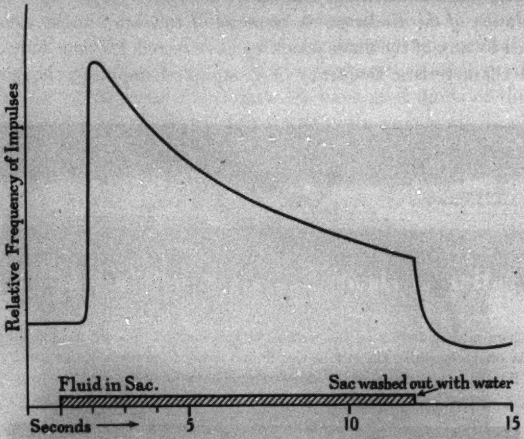

Fig. 11. Smoothed curve to show evolution of the discharge illustrated in Fig. 8.

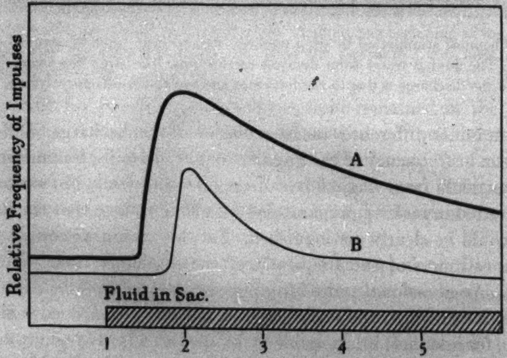

Fig. 12. Smoothed curves to show evolution of discharges with strong and weak stimulation. Curve *A* shows the response to a drop of fluid from decayed earthworms. *B* shows the response to a drop of the fluid diluted with 10 vol. of water.

the discharge often takes ½ sec. or more to reach its maximum, and after the maximum there is a decline which is rapid at first and then slows down so that the increased activity is still perceptible 10 sec. after the start.

When the stimulating fluid is filtered and diluted with 5–20 times its volume of water the discharge is much less intense and the increased activity seems to last for a shorter time, becoming inappreciable within a few seconds. Curves to show the effects of concentrated and diluted fluids are given in Fig. 12.

Latency. Even with a strong stimulus the latency of the discharge is often a second or more (cf. Fig. 13) and may be as great as 5 sec. In many (but not in all) of the preparations the latency has been greater when the stimulating fluid has been diluted. A longer delay with weaker stimulation is reminiscent of the optic nerve discharge, but the great variations from one preparation to another suggest that the delay is due in part to the time taken by the fluid to penetrate to the receptor cells, e.g. through a layer of mucus. On this hypothesis an increasing secretion of mucus would account for the fact that the latency may increase progressively with the time which has elapsed since the preparation was made. Differences in the rate of penetration would also explain why the latency may be much longer for one fluid than for another, although the discharge, when it does appear, is no less intense. Thus in one preparation the latency for the earthworm fluid was 3 sec. and that for the alligator fluid less than 1 sec.; yet the response was if anything greater after the longer latency. On the other hand, if a long delay is due to the long time taken by the fluid to reach the receptor cells, we might expect that the latency would be modified by washing the sac repeatedly or by injecting the fluid instead of allowing it to drop into the sac. As neither procedure has much effect it cannot be said that the hypothesis of slow penetration is more acceptable than that of slow reaction time in the receptors or in the synapses of the olfactory bulb.

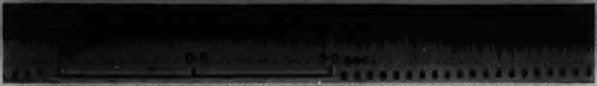

Fig. 13. Latency of discharge with chemical stimulation. Discharge produced by a drop of fresh liver extract (filtered). The moment of application is shown by the downward excursion at the beginning of the record. The discharge begins after a latency of about 1 sec.

After-effects of stimulation. By mechanical stimulation the organ can be made to respond several times in rapid succession (i.e. at intervals of a second or less), though it is soon injured beyond recovery. With chemical stimulation the after-effects are much more variable. In one preparation which was unusually active and survived for nearly 2 hr., repeated discharges could be produced at intervals of 2–3 sec. (the shortest time in which the fluid could be washed away and a fresh drop placed in the sac). In this preparation, even after a long period of stimulation, removal of the fluid merely reduced the activity to its initial "resting" level. In several others, however, each period of stimulation made the organ insensitive to a second stimulus (by the same or by a different substance) for an interval of 5–10 sec., and during this interval the resting activity was abolished or greatly diminished.

The silent period following activity is illustrated in Fig. 14. The first record shows the vigorous discharge caused by placing a drop of the alligator fluid in the sac. The second shows the reduction in activity to far below the resting level when the fluid is washed out with water. The third shows the discharge 20 sec. later after it had gradually returned to the initial "resting" value. The return of the resting discharge is not delayed by washing the sac repeatedly with water: it is not due, therefore, to the accumulation of some stimulating material in the sac. Presumably it is due to the gradual return of excitability in the receptors following a refractory state. The curves in Fig. 15 show that the return to normal is much quicker when the stimulation has been less intense.

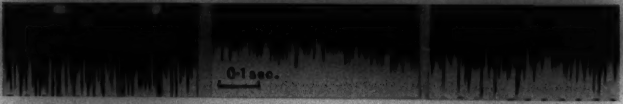

Fig. 14. Failure of resting discharge following stimulation. Small filament of olfactory stalk. Stimulation by decayed alligator fluid. A, during stimulation B, 3 sec. after washing out sac with water C, 20 sec. later. Resting discharge back to initial value.

Although the removal of the chemical stimulus usually causes an immediate reduction or suppression of the discharge in the olfactory stalk, there is one stimulating material which has given a different result. This is a decoction of aniseed made by boiling the powdered seed in water. The fluid has a considerable stimulating effect, but if it remains in the sac the discharge becomes inappreciable after a few seconds and there is then a definite renewal of activity (to well above the resting level) when the fluid is washed out with water (Fig. 16). This "off" discharge with water following aniseed has been recorded on many occasions with the constant flow as well as with the drop method of stimulation.

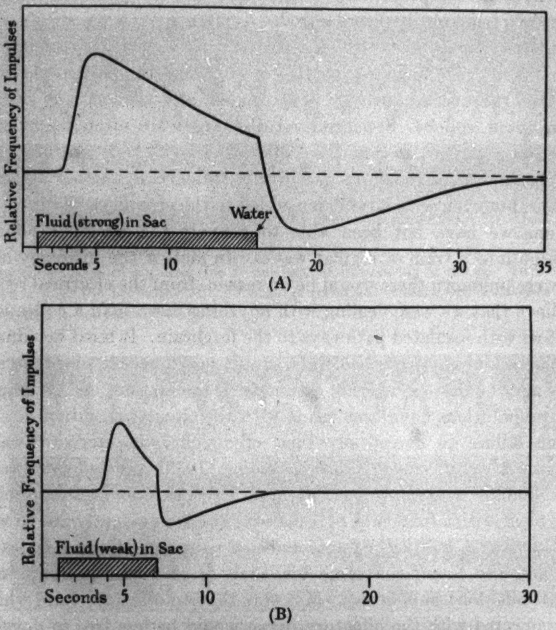

Fig. 15. Curves to show after-effects of strong and weak stimulation.

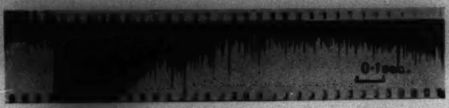

Fig. 16. "Off discharge" with injection of water following stimulation by decoction of aniseed.

Washing out the sac with water has also given a discharge in many preparations at the beginning of an experiment. If the sac is not touched during the dissection there is often very little of the normal resting activity when the preparation is first examined. A drop of water placed in the sac will then cause an intense discharge which afterwards declines to the steady resting level. Both this and the aniseed effect may be due in part to mechanical causes, for sticky particles which have become adherent to the receptor surface might be washed off by the water leaving the surface temporarily disorganized.

Absence of synchronous activity. In view of the fact that the impulses in the olfactory stalk come from the nerve cells in the bulb, it is remarkable that the discharges have never shown any tendency to occur in synchronized volleys. Synchronized discharges are often found in the optic nerve (particularly in the "off" discharge) and in other nerves which lead from a group of nerve cells which can become associated together, but even with strychnine added to the cranial cavity in various dilutions we have not been able to convert the irregular olfactory discharge into a train of regular waves. In fact, if the histology of the bulb were unknown there would be no reason, from the electrical records, to believe that we were dealing with anything more than a collection of receptors with insulated pathways to the forebrain. It must be admitted that the lack of an adequate oxygen supply might account for this result, and it may be that evidence of summation, interaction, etc., will appear when preparations have been made with the circulation intact.

The failure to detect any kind of synchronous activity was an unexpected result, since Gerard & Young [1935] have shown that the frog's olfactory bulb develops rhythmic electrical waves after excision. These recur with a frequency of 3–5 a sec., and are evidently due to waves of activity in a number of interconnected neurones. Gerard states that Hoagland has found the same tendency to rhythmic activity in the isolated olfactory bulb of fish. It is true that an olfactory bulb which is still connected with the olfactory organ would be less free to develop a synchronized beat, since the different receptors might impose different levels of excitation on the mitral cells. But whatever the explanation may be the fact remains that we have not detected any sign of a synchronous response in the olfactory stalk, either without stimulation or with intense stimulation of the olfactory receptors.

Rhythmic activity in the forebrain. Although it has little connexion with the olfactory discharge there is one very clear example of a synchronous beat which is often observed. It is a rhythmic electrical activity occurring in the forebrain and producing large potential changes at 2–4 a sec. Examples are shown in Fig. 17. As one of the electrodes usually makes contact with the forebrain, the potential waves, though not due to the olfactory stalk, are none the less able to appear in the record, but if the leads are confined to the stalk the waves no longer appear. Their origin is also shown by the fact that they continued in the forebrain after the olfactory stalk has been cut through, and that they are abolished by crushing the forebrain and altered by less extensive damage to it.

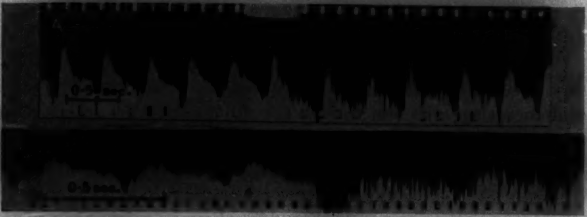

Fig. 17. Rhythmic potential waves occurring in the forebrain. In A the rhythm is unaffected by a discharge of impulses due to mechanical stimulation of the sac. In B the first wave after the beginning of the discharge (chemical stimulation) is delayed but the rhythm is not otherwise affected.

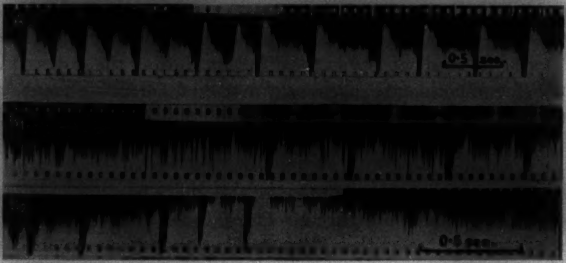

Fig. 18. Forebrain rhythms modified by olfactory discharges. In A the frequency of the waves is reduced during the period of stimulation. In B_1, another preparation, the signal indicates the washing out of the sac with water. The waves only occur during this period, when the discharge is greatly reduced. B_2 shows the end of the washing and the failure of the rhythm.

When the rhythmic potential charge has appeared, it is usually quite unaffected by a discharge of impulses reaching the forebrain from the olfactory stalk. This is illustrated in Fig. 17 A. Occasionally an olfactory discharge seems to start or stop the forebrain rhythm or to upset the regularity for a few beats. Some examples of this are given in Fig. 18, but it would be misleading to suggest that such examples are common, for as a rule the forebrain rhythm seems to be quite independent of the olfactory system. This fact and the large size of the potential waves make it highly probable that the rhythm only occurs when the neurones of the forebrain have become associated with one another to an abnormal degree. They have become scarcely accessible to impulses reaching them by the olfactory pathway, but if an olfactory discharge has any effect at all it is to slow the rhythm or else to abolish it completely.

Olfactory discharges in carp and tench

We have fewer data from the carp and the tench, since the preparations do not survive so well and the action potentials are smaller, but we have enough to show that the olfactory discharges are of much the same type as in the catfish. There is some resting activity; a greatly increased discharge is produced by mechanical stimulation of the organ, and chemical stimulation gives a discharge which may continue for 10 sec. or more if the fluid is left in the sac. As in the catfish a decoction of aniseed gives a definite "off" discharge, but our records have not been clear enough to show whether there is anything in the nature of a silent period after excitation by other chemical stimuli.

The list of solutions which do and do not stimulate appears to be the same for the carp and tench as for the catfish, and there has been no sign of synchronized activity in the different fibres of the stalks. In fact we have found nothing to suggest any marked difference in the reactions of the olfactory system in the different fish.

Nature of stimulating substances

Table I gives a list of the substances which have been found to give an olfactory discharge in the catfish and of those found to have no effect. They were selected because of their characteristic smell (for man) or because there was reason to suppose that they might act as olfactory stimuli to fish. All of them were in emulsion, suspension or solution in water.

The list of substances which have been found to stimulate agrees fairly well with the list compiled by Olmsted as the result of his observations on the behaviour of intact catfish. He found that the fish approach a bag of earthworms because of a slimy protein substance secreted from the worm's skin. Other slimy animal products (human saliva, frog's eggs) were also found to act as stimuli. Extracts of liver and of blood were particularly effective, but extracts of frog muscle and of cheese were not.

In the present experiments extracts of fresh liver and blood have produced olfactory discharges, and so has the water in which earthworms

TABLE I

Substances giving a definite olfactory discharge:
- Watery extract of fresh earthworms
- ,, fresh liver (rabbit and frog)
- ,, fresh blood
- Water in which live earthworms have been placed
- Fresh decoction of aniseed and of ants' eggs
- Watery extract of decayed earthworms
- ,, ,, frog muscle
- ,, ,, liver
- ,, ,, alligator tissues (head)
- ,, ,, gelatin
- ,, ,, blood

Substances giving no olfactory discharge:
- Emulsion of oil of cloves
- ,, oil of cedar
- ,, oil of rhodian
- Tincture of asafoetida (1 drop in 2 c.c. water)
- ,, valerian ,,
- Rose water
- Watery extract of fresh frog muscle, catfish muscle and skin
- Milk
- Starch solution
- Suspension of cheese

Substances which have occasionally given a discharge:
- Saliva
- Fresh gelatin solution (0·2%)
- Serum (rabbit)

have been placed. Saliva has only given an occasional discharge. The chief difference is that the most intense discharges have been produced by decayed animal matter, whereas Olmsted found that on the whole putrefaction reduced the attractiveness of the material to the fish. The simplest explanation of the discrepancy would be to suppose that the catfish, like man, is not necessarily attracted by every substance which stimulates the olfactory organs, but there is little evidence either for or against this supposition. Another possible reason for differences between Olmsted's list and ours is that to produce a recognizable discharge in the olfactory stalk the stimulating fluid must contain a relatively high concentration of active material. The catfish swimming in an aquarium is attracted to a bag of earthworms several feet away, and this reaction is presumably due to occasional threshold stimulation from small traces of active material which have diffused away from the bag. In electrical records, however, the presence of the resting discharge in the olfactory stalk makes it impossible to detect the very small increase in activity which would be caused by a threshold stimulus—with the result that all our findings relate not to threshold stimulation but to stimulations intense enough to give a distinct outburst of action potentials.

The particular stimulating substances present in the fluid from decayed animal tissues have not been identified. All that can be said is that the effect is not due to an acid or alkaline reaction or to the presence of indole in solution. But it is noteworthy that the fluids which are the most effective stimulating agents all contain solid particles and lose much of their effectiveness when they are filtered. This applies not only to the fluids from decayed tissues but to extracts of liver, blood, etc. Filtration (through ordinary filter paper) does not completely abolish the stimulating power of the fluid, but as a rule the filtrates remain faintly opalescent. In fact we have not found any substance which dissolves freely in water to give a solution which will stimulate the olfactory organs without causing permanent damage. Strong acids or alkalies produce a brief discharge as they destroy the organ. Sodium citrate and oxalate solutions do not.

Discussion

It is satisfactory, though not surprising, to find impulse discharges in the olfactory stalk while the olfactory organ is stimulated, and it must be admitted that the present observations have not gone much further. But there are three findings which were not anticipated and are therefore more interesting. They are, first, the reaction of the olfactory organ to mechanical stimulation; second, the importance of solid particles in the fluids which stimulate chemically, and third, the absence of any synchronous activity in the different fibres of the olfactory stalk.

Mechanical stimulation is probably effective because it injures the olfactory receptors and produces a breakdown of the surface layers. The delicate hairs which protrude from the ends of the receptor cells would be injured by very slight pressure. But it is interesting to find that mechanical deformation, or injury, will set up such an intense olfactory discharge, for this raises the question whether small particles of decayed tissue may not excite the receptors by causing similar though less complete surface changes. It is possible, for instance, that a particle brought in contact with one of the hairs might cause a local disintegration of the surface through a combination of chemical and mechanical action, the chemical action being due to substances adsorbed on the surface of the particle and so present locally in very high concentration. At all events, for chemical stimulation it is probably essential that there should be differences in the concentration of the active material in different parts of the receptor surface, and the concentration gradients would certainly be sharper if the material were attached to solid particles than if it were present in solution.

The third finding—the absence of synchronized activity—might perhaps have been anticipated on structural grounds. The olfactory receptors are connected to the mitral cells on the bulb by sharply defined synaptic structures, the glomeruli, with no lateral connexions. Several receptors lead in this way to one mitral cell, but the dendrites of adjacent mitral cells do not form a network through which interconnexion might be established. It is true that there are some additional neurones with laterally directed dendrites which might establish connexion between one mitral cell and another, yet the general arrangement suggests that the pathway from each group of receptors to the corresponding nerve fibre of the olfactory stalk is more highly insulated than, for example, the pathway from a group of rods or cones to a fibre of the optic nerve.

A large number of independent pathways between the receptors and the brain would certainly favour the discrimination of different olfactory stimuli. There is no clear evidence that the catfish can distinguish one smell from another, but it would be difficult to account for the large size of the olfactory organ if it has no discriminative powers, and in warm-blooded vertebrates the olfactory system can certainly indicate quality as well as intensity. The mechanism of discrimination probably depends on the production of characteristic patterns of excitation by different chemical stimuli, patterns which might be determined by various physical and chemical gradients in the organ. If these patterns are to be reproduced faithfully in the integrating regions of the brain there must be, as we have found, a reasonable degree of insulation between the different pathways through the bulb, as well as a large number of pathways.

These arguments have already led some way beyond the facts; the next step must be to collect more information, if possible by records from single nerve fibres of the olfactory stalk or from small groups of fibres. This should be possible in spite of the great delicacy of the fibres. A further advance would be made if a method could be devised for recording the impulses in the non-medullated fibres of the olfactory nerve. This, however, is a much more difficult technical problem, and at present there is not much prospect of solving it.

SUMMARY

1. The mode of action of the olfactory organs can be studied by recording impulses in the long olfactory stalk of the catfish, carp or tench. The nerve fibres of the stalk are medullated and are separated by only one synapse from the receptor cells.

2. The olfactory organs in these fish are sensitive to mechanical as well as to chemical stimulation. Chemical stimulation is brought about by various animal products and is most effective when the fluid contains solid matter in suspension.

3. When a drop of stimulating fluid is placed in the olfactory sac the discharge begins after an appreciable latency (from 0·5 to 5 sec. or even longer), rises rapidly to a maximum and then declines more slowly. With a concentrated fluid the discharge may continue for many seconds, but it ceases at once when the sac is washed out with water. The rate of adaptation of the olfactory system cannot be classed either as very fast or very slow.

4. A period of stimulation which has produced a considerable discharge is usually followed by a period of inactivity lasting 5–20 sec. or more. During this period the organ is insensitive to a second stimulus and the normal resting activity is greatly reduced.

5. Decoctions of aniseed give an exceptional result, for with these there is a renewed discharge when the fluid is washed out with water. This "off effect" may be the result of mechanical stimulation.

6. We have found no evidence of synchronous activity in many fibres of the olfactory stalk. Large rhythmic potential waves often appear in the forebrain, but in the olfactory bulb the connexions between receptors and initial cells appear to form a series of independent pathways.

7. We have no evidence as to the possible discrimination of different chemical stimuli by these olfactory organs. The discharges recorded from the whole nerve would be unlikely to show distinguishing features and we have not yet succeeded in making preparations with very few fibres.

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STUDIES ON THE CHEMICAL PROCESSES WHICH OCCUR IN MUSCLE BEFORE, DURING AND AFTER CONTRACTION

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In 1937 I described [Dubuisson, 1937*b*, *c*] a method for measuring the alterations in *p*H which occur at the surface of a muscle in the course of its activity. The method, however, was not entirely free from error: I was recording not only the changes in *p*H, but also variations in tissue polarization. Later, the technique was sufficiently improved to permit the study of post-contractile phenomena [Dubuisson, 1937*d*]. The latter are of special interest, since the formation of acid or alkaline products of non-instantaneously reversible reactions can be followed by the variations in hydrogen-ion concentration. By this means we have been able to compare the quantitative data from this recording technique with the results of chemical analyses made immediately after the experiment on the same muscles, and we have found an excellent correlation [Dubuisson & Schulz, 1938]. These investigations have demonstrated that this method of measuring alterations in muscle reaction allows not only a qualitative determination but also a quantitative analysis of those processes which leave post-contractile residues, and that these products are the result of known chemical reactions: the hydrolysis of phosphagen, the esterification of phosphates, and the formation of lactic acid.

With the present improved technique, it is possible to gain an insight into the changes in reaction which accompany contraction, in smooth and striated muscles.

METHOD

Principle of the technique. The *p*H of a Ringer solution containing known quantities of sodium bicarbonate and CO_2 is given by the Henderson-Hasselbalch equation:

$$pH = pK_{CO_2} + \log \frac{[NaHCO_3]}{[CO_2]},$$

where pK_{CO_2} is a constant (6·26 in the case of a Ringer solution). The concentrations of $NaHCO_3$ and of CO_2 are expressed in equivalents. If we place a muscle in a Ringer solution whose bicarbonate content is identical with that of the normal muscle (about 2×10^{-2} *M*), and if the immersion is sufficiently prolonged to allow the establishment of an equilibrium between the CO_2 in the muscle and in the solution, the internal *p*H of the muscle should become equal to that of the medium. If, at this time, the solution is discarded, there still remains, adhering to the muscle surface, a thin liquid film. A glass electrode placed on the tissue enables one to measure the *p*H of this film of Ringer. If on contraction an acid substance is formed in the muscle, it reacts with the tissue's alkaline reserve, consisting largely of bicarbonates, and liberates an equivalent quantity of CO_2. This excess of CO_2 concentration in the muscle over that of the surrounding atmosphere brings about a diffusion of CO_2 from the muscle to the exterior. The film of Ringer which coats the organ thus becomes a region across which CO_2 transpires; however, being under the glass electrode, the gas cannot diffuse into the atmosphere and continues to accumulate in the Ringer film until a new equilibrium is obtained between the muscle's CO_2 concentration and that of the film. As a result of this CO_2 accumulation, a diminution of *p*H, as measured by the glass electrode, takes place; this enables a calculation, by means of the Hasselbalch equation,[1] of the amount of CO_2 which has accumulated. Conversely, the liberation of a basic substance in the muscle will diminish the CO_2 concentration in the Ringer film under the electrode.

In order to relate the variation in CO_2 concentration found in the Ringer film to the equivalents of acid or base liberated in the muscle, two corrections are introduced: (*a*) one which takes account of the solubility of CO_2 in the muscle, and of the quantity of water actually contained therein; (*b*) another which allows for the presence, in the muscle, of substances which can "bind" CO_2. It has been assumed [Dubuisson & Schulz, 1938] that (*a*) the space in which CO_2 can dissolve = muscle weight multiplied by 0·85; (*b*) the retention factors are those established by Meyerhof, Möhle & Schulz [1932].

Glass electrode and contact electrode. As before, the glass electrode is of the MacInnes type [MacInnes & Belcher, 1931]: at the lower end of an ordinary thick-walled glass tube a membrane of Corning no. 015 glass [MacInnes & Dole, 1930] is sealed. In our electrode this membrane is from 1 to 2×10^{-2} mm. thick; its surface is about 8 mm.^{2} The D.C.

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[1] If $[NaHCO_3] = 2 \times 10^{-2}$, then $\log [CO_2] = 4{\cdot}56 - p\text{H}$.

resistance of this membrane is about 700 MΩ. This resistance is principally a polarization resistance (resistance plus capacity): it depends upon the frequency of the current [MacInnes & Belcher, 1931]. The interior of the glass tube is filled with a solution of 0·1 *N* HCl. In this is immersed a d'Arsonval silver chloride electrode, prepared according to Brown [1934].

The contact electrode is composed of a tube filled with Ringer solution without bicarbonate in which is also immersed a wire of chlorided silver. The lower part of the tube is plugged with cotton wool.

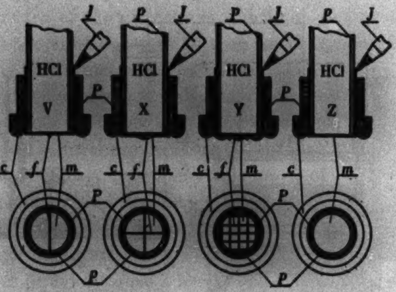

Fig. 1. Schematic representation of four types (V, X, Y and Z) of glass electrodes. *m*, glass membrane; *p*, wall of ordinary glass; *c*, rubber sleeve whose margin is tucked up; *f*, cotton thread; *J*, contact electrode; *P*, collar of filter paper.

If the contact electrode is placed on a part of the muscle at a distance from the glass electrode, the latter records, during a contraction, the *p*H variations in the Ringer film under the glass membrane, simultaneously with other potential variations of physiological origin (action potentials, fluctuations in tissue polarization, etc.) which arise between these two points of the muscle. Theoretically, the only way to eliminate the potentials which are not due to modifications of the hydrogen-ion concentration is to place the contact electrode and the glass electrode *at the same point on the muscle.* We have devised numerous arrangements allowing us to achieve this result, in principle. In Fig. 1 are found a number of diagrams which illustrate these combinations. The type V electrode has already been described [Dubuisson, 1937*d*]. One finds that arrangements which eliminate the recording of the action potentials are but mediocre *p*H indicators. A series of controls has indeed shown us that from this point of view the "figure of merit" of the different electrodes is far from being identical. Electrode Y was the most sluggish, giving small spread out tracings, whilst electrode Z was the fastest, giving a high sharp record. The thickness of the layer of Ringer solution under an electrode of type V, X or Y is determined by the thickness of the threads which make up the woof; whereas under electrode Z it is doubtless reduced to an extremely thin film, the effect of the pressure of the glass electrode on the muscle being to express the excess of fluid, and to establish as perfect a contact as possible between the glass membrane and the deformable muscular aponeurosis. The pellicle of Ringer solution which lies interposed between them is probably reduced to a "film" in the physical sense of the word.

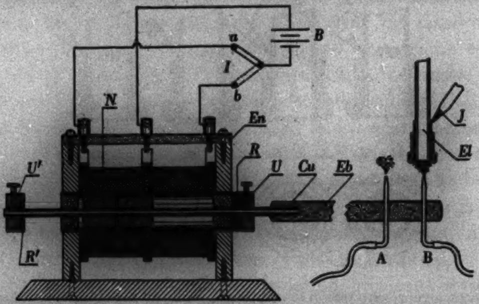

Fig. 2. Diagram of the apparatus for instantaneously modifying the pH of a solution in contact with a glass membrane. *Cu*, brass rod, extended by an ebonite rod *Eb*, the latter penetrated by the capillaries *A* and *B*. *N*, iron cylinder. *R* and *R'*, felt discs. *U* and *U'*, collars acting as stops. By the operation of interrupter *I*, current from battery *B* is sent through either winding, which allows either the liquid from capillary *A*, or that from capillary *B* to be brought under the glass electrode *El*. *J*, contact electrode.

If electrode Y is slow, it is because the volume of liquid which separates its active membrane from the muscle surface is too great; the establishment of equilibrium between the muscle CO_2 and that of the liquid immediately subjacent to the glass membrane requires too long a time. The importance of the thickness of this liquid layer in the speed of the glass electrode's response is enormous, as can be experimentally demonstrated in the following manner: capillaries *A* and *B* of Fig. 2 are supplied with CO_2–O_2 mixtures, the concentration of CO_2 being, for example, slightly weaker in *A* than in *B*. Manipulation of the interrupter *I* (with a speed which depends only upon the electromagnetic properties of the system) allows a jet of gas either from capillary *A* or capillary *B* to pass to the glass electrode. A droplet of bicarbonate was suspended under the glass electrode. By touching the droplet with filter paper its volume could be progressively diminished and the speed of response of the electrode could be followed with the galvanometer. Whereas the response is rapid with a glass membrane only slightly moistened, it requires as long as 30–60 sec. when the liquid suspended from the glass electrode has the form of a droplet which has a thickness of 0·2–0·3 mm. The electrode which, on the muscle, will give the most faithful results, will then be one whose glass membrane lies directly in contact with the tissue: this will be an electrode of type Z.

To gain an idea of the distortions which can be introduced into the records (1) by the time constant of such an electrode, (2) by the time constant of the electrometer, (3) by the time constant of the galvanometer (critically damped), we have used the same set-up as that represented in Fig. 2, but passed solutions of different pH through tubes *A* and *B*. These solutions, composed of mixtures of primary and secondary phosphates, were contained in Mariotte bottles whose height could be readily regulated. They were adjusted so that each jet of liquid attained a height of 2–3 cm. By adjustment of the interrupter *I*, the glass membrane could be brought quickly into contact with either solution, and we could measure, with the galvanometer, the time taken to reach the new equilibrium. Fig. 3 is a photograph of the result of such a procedure. It shows that the glass membrane, the electrodes, the electrometer and the galvanometer used are such that about 4 sec. are required for an instantaneous variation in pH under the glass membrane to be translated into a steady final deflexion on the photographic paper.

Fig. 3. Speed of response of glass electrode, electrometer and galvanometer to a sudden change of pH.

It now remains to show that this type Z electrode is not capable of responding to action potentials. The potential of the glass electrode is that of the muscle surface covered by the membrane; the potential of the contact electrode is that of a circular zone circumscribing the glass membrane. Theoretically, these conditions are very unfavourable for recording changes in tissue polarization. In practice, one can be assured that such an arrangement of the electrodes does not allow the reception of action potentials by constructing an identical model, but one in which the glass membrane is broken and replaced by a bit of cotton wool. Such an electrode has never shown any potential following supermaximal stimulation of striated or smooth muscle.

To summarize, the results of numerous tests on type Z electrode show that it is protected from potentials which are not due exclusively to hydrogen-ion concentration, and yet possesses the most remarkable properties for recording changes in pH which appear in the film of Ringer solution covering the muscle.

Electrometer. This instrument is the same as that described in another publication [Dubuisson, 1937 *c*], except that the valve Pliotron F.P. 54 was replaced by a model T Géco-valve [Dubuisson, 1937 *d*]. By leaving our electrometer switched on *permanently*, it becomes extraordinarily stable. In dry weather no variation in anode current is observable: when it is very humid the variation is never greater than 0·1 mV. in 2 or 3 min.

Galvanometer. A Siemens galvanometer is used, critically damped, which in our circuit gives a deflexion of 35 mm./mV., at 1 m. distance. This easily allows the reading of $\frac{1}{35}$ mV., which is equivalent to about $\frac{1}{2000}$ pH.

Recording of the mechanical contractions. Since it is well to avoid any movement of the muscle during its contraction, our experiments were carried out only under isometric conditions. The maximum shortening of the muscle never exceeded one-eightieth of its length, either for smooth or striated muscle. As this system of electrodes and muscle is necessarily placed in a shielded box, we have recorded the isometric contractions by an indirect optical method. The muscle is previously attached to the membrane of a Marey tambour connected pneumatically to another Marey tambour (Fig. 4). The membrane of the first tambour is made of a piece of rubber cut from an automobile inner tube, whilst the membrane of the second tambour is of extremely thin rubber. The pull of the muscle on the first membrane causes a reduced pressure on the second; this is recorded optically (for other details see Fig. 4).

Preparation of the muscles and stimulation. Our experiments were carried out on the gastrocnemius, the sartorius, and the stomach of frog (Hungarian frog) and tortoise. The gastrocnemius and sartorius were prepared with the sciatic nerve and stimulated indirectly. The stomach preparation consists of a ring 1–2 cm. wide. This ring is attached in the chamber as shown in Fig. 5 (the mucous membrane being turned inside) and stimulated directly through platinum electrodes. The excitation of these muscle or nerve-muscle preparations is brought about with a variable induction coil (single shocks or tetanization). After dissection, all muscles are immersed for 30–90 min. in a Ringer solution containing 2×10^{-2} M $NaHCO_3$ through which oxygen containing various proportions of CO_2 is continually bubbled. The solution is then removed and the tissue enclosed in an atmosphere of nitrogen gas with an equal percentage of CO_2.

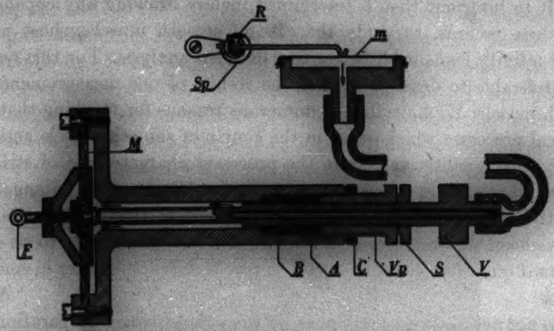

Fig. 4. Diagram of double tambour for pneumatic transmission and recording of isometric contraction. The muscle is attached at one of its ends to the ring *F* which is fixed to a thick rubber membrane *M*. At each muscle contraction, the membrane *M* becomes deformed, thus producing a movement of air (in the direction of the arrows in the diagram). The net result is a depression of the rubber membrane of the second tambour, which induces a movement of the concave mirror *R*, about its axis of rotation. A spiral insures permanent contact of the lever of this mirror with the membrane *m*. The sensitivity of the instrument can be adjusted by the knob *V* which varies the initial tension of the membrane *M*. *B*, padding.

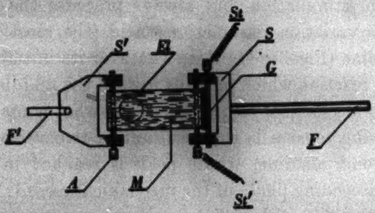

Fig. 5. Sketch of the holder for attaching a ring of frog or tortoise stomach for isometric contraction. *M*, ring of muscle, held by rods *A* and *St'*. Rods *St* and *St'* are platinum, and conduct the stimulation current. The contact between the electrode *St* and the external wall of the muscle is insured by a wad of cotton soaked with Ringer solution (*G*). The holder *F'* is an integral part of the glass chamber in which this apparatus is contained. The rod *F* is attached to the ring *F* of the pneumatic tambour in Fig. 4. *El* marks the region where the glass electrode is placed.

Results on smooth muscle

A. *Qualitative.* We worked, as a matter of fact, on striated muscle before studying smooth muscle. But the records of *p*H variations occurring *during the contraction* of striated muscle appeared at the outset so difficult to interpret that I determined, before drawing any conclusion from these records, to study the case of smooth muscle whose much slower activity lends itself better to a detailed analysis. For this reason it is preferable to describe at first the results of our measurements on smooth muscle. We have indeed numerous reasons for thinking that the chemical processes which occur in the course of smooth muscle activity do not differ qualitatively from the processes characteristic of striated muscle: all work is in agreement on this point [Evans, 1925; Mangold & Schmitt-Krahner, 1926; Eggleton, 1934; Bozler, 1930; Dworaczek & Barrenscheen, 1937; Meeraus & Lorber, 1937]. There appear to be only important quantitative differences, the reactions being weaker in smooth muscle.

It is not necessary to distinguish between the stomach preparations of the tortoise and those of the frog: both give quite similar records, qualitatively and quantitatively.

In all our photographs the pH variations begin *before* the isometric contraction. The duration of the interval between the beginning of stimulation and initiation of pH changes depends upon the latent period of the tissue, which can differ greatly from one preparation to the other, even for stimuli of equal duration. The pH change starts from 2 to 8 sec. after the beginning of stimulation, but always precedes the contraction by 2 to 4 sec. This variation is sometimes acid (the most frequent case), sometimes alkaline (Figs. 6–8). But, whatever its initial direction, it is always the acid reaction which prevails toward the middle of the ascending phase of the mechanical record. In short, what is manifested during the phase of rising tension is the resultant of two variations in pH of opposite sign: one, alkaline, whose peak is reached in the very first instants of the ascending phase of the mechanical record; the other, acid, larger in fresh muscles, and whose maximum appears to be reached in general during the first two-thirds of the period of increasing tension. On our drawings and photographs we have designated the alkaline variation by the letter (*a*) and the acid variation by the letter (*b*). These two phases appear rapidly reversible: the study of a great number of records has led to the conclusion that the process which determines variation (*a*) returns to its initial state toward the middle of the period of increasing tension and that that which determines variation (*b*) returns to its initial state during the relaxation period.

The acid variation (*b*) is, for a given initial pH of the muscle, greater when the muscle is fresh than when fatigued: it is, in any particular muscle preparation, proportional to the tension developed. Its maximum is sharp and early to the same extent that the contraction is less sustained. It is quite difficult to say whether such is also the case for variation (*a*), the amplitude of the latter being too small in comparison with (*b*) to enable one to make a definite statement on this point. It appears only that fatigue affects the amplitude of (*b*) more profoundly than that of (*a*).

One can then detect at the surface of smooth muscle two alterations in reaction of opposite sign, which appear completely reversible in anaerobic contraction, one alkaline, whose summit is found in the very early period of contraction (*a*), and the other acid whose peak is situated in the first two-thirds of the period of increasing tension (*b*). (An acid period was not observed in two out of 140 fresh muscles, although the contraction appeared normal.)

Fig. 6. Frog stomach, normal muscles, not fatigued. Above, at the left, two superposed successive contractions (*I*) of the same muscle, and the corresponding *p*H variations, showing the phases (*b*), (*c*) and (*d*) (the alkaline phases (*a*) do not appear clearly here). Initial *p*H 7·45. Temp. 19·5° C. Above, on the right, *sustained contraction*. After the alkaline phase (*a*), an acid phase (*b*) is observable, *which is large and whose return is slow.* Initial *p*H 7·20. Temp. 20·6° C. Below, two superposed successive contractions of the same muscle and the corresponding variations in *p*H, distinctly showing the alkaline phases (*a*) and (*c*) and the acid phases (*b*) and (*d*). One of the contractions is slightly stronger than the other; phase (*b*) of the first is also larger. Initial *p*H, 7·45. Temp. 21·5° C. Direction of acidification, toward the bottom; alkalization toward the top.

Later, an alkaline peak appears at the beginning of relaxation (*c*). Then follows a post-contractile acidity (*d*). Altogether there are four phases.

According to the conditions of the experiment, certain phases can assume a greater or lesser importance, others may remain unaffected, or amplified, so that it is principally by varying the experimental conditions that one can form an opinion of the evolution and significance of these

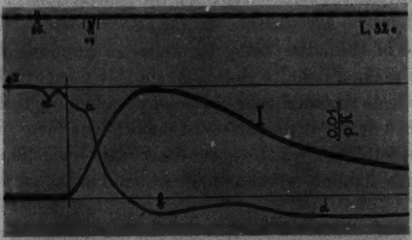

Fig. 7. Frog stomach, normal muscle, not fatigued. After the stimulation artefact (*st*) one sees the acid phase (*b*) large, and the alkaline phase (*a*) weaker. From the curve *I* (isometric contraction) it may be noted that the contraction is well sustained and the relaxation slow, conditions in which phase (*b*) is long and phase (*c*) small. Initial *p*H, 7·32. Temp. 21·5° C. Acidification toward the bottom, alkalization toward the top.

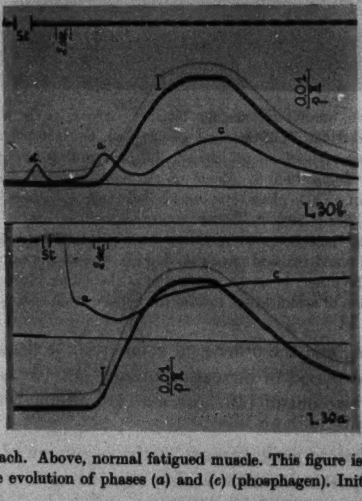

Fig. 8. Frog stomach. Above, normal fatigued muscle. This figure is included because it shows well the evolution of phases (*a*) and (*c*) (phosphagen). Initial *p*H, 7·10. Temp. 21° C.

Below, from the same stomach, but muscle was poisoned with monoiodoacetic acid. Phase (*c*) is maintained at its maximum (no resynthesis of creatine phosphoric acid). Initial *p*H, 7·22. Temp. 21·2° C. *I*, isometric contraction. *St*, stimulation artefact. Acidification below, alkalization toward the top.

changes in reaction. The problem of the discrimination of these four phases can be approached by comparing: (1) records taken of muscles previously poisoned by monoiodoacetic acid, and of normal muscles; (2) records of muscles whose initial *p*H differed.

(1) It is known that when a muscle is completely poisoned, there is no formation of lactic acid and no resynthesis of phosphocreatine; the hydrolysis of phosphagen leads consequently to a non-reversible alkalization, diminished only by the acidity brought about by the esterification of phosphates (monohexosephosphoric ester and dihexosephosphoric ester). In records of muscles poisoned with monoiodoacetic acid (1 in 12,000 for 60 min.) the variations (*a*) and (*b*) appear unaffected, variation (*c*) continues, ending in a plateau, and there is no post-contractile acidification (*d*) (Fig. 8).

These first statements would then permit us to relate the change in reaction (*c*) to the hydrolysis of phosphagen and the alteration in reaction (*d*) to the formation of lactic acid.

(2) We have seen previously that in striated muscle the progressive alkalization of the muscles during fatigue is more or less considerable, depending upon the initial *p*H of the muscle. This comes about from the fact that the curve of the electrotitration of phosphagen [Meyerhof & Lohmann, 1928*b*; Meyerhof, Schulz & Schuster, 1937] has a special form such that for a given quantity of hydrolysed substance, the basic equivalents liberated depend on the *p*H. In smooth muscle, while the post-contractile acidification attributed to lactic acid (variation (*d*)) appears unaffected by the initial *p*H (the equivalence factor for lactic acid = 1 in the entire range of physiological *p*H's), the alkalization (*c*), whose maximum occurs at the beginning of relaxation, bears an inverse relationship to the initial *p*H. This result is established by measurements made between *p*H 7·40 (2% CO_2) and 6·80 (16% CO_2). We were not able to go lower than 6·8; we have regularly observed that it is not possible to maintain a frog or tortoise stomach in good condition in CO_2 concentrations above 16%.

This also enables us to relate the variation in reaction (*c*) to phosphagen $\left(\frac{\text{mg. } H_3PO_4}{\text{c.c. } CO_2}\right.$ varies from 13 at *p*H 7·2 to 5·85 at *p*H 6·20$\left.\right)$.

It appears, then, from the comparison of records obtained with normal muscles, and those poisoned by monoiodoacetic acid, as also from the comparison of records of muscles at different initial *p*H, that the hydrolysis of phosphagen makes itself evident at the surface of a muscle by a change in reaction (phase (*c*)) which has its maximum at the beginning of relaxation [cf. Dubuisson, 1936, 1937 *a*] and that the formation of lactic acid corresponds to phase (*d*).

Of the four phases which we have delineated, this last is by far the slowest: beginning at the end of the period of increasing tension, it evolves slowly toward a maximum which is attained only several minutes after the end of the contraction.

With regard to variation (*b*) (and perhaps also variation (*a*)) there obtains likewise a relation between its amplitude and the initial *p*H of the muscle. In this case, however, it is the reverse of what holds for phosphagen (*c*): the lower the *p*H, the smaller the amplitude of (*b*). This fact leads us to think that the equivalence factor of the substance whose decomposition provokes process (*b*) is increased as the *p*H is made more alkaline. I may say here immediately that amongst the chemical processes known in muscular contraction (smooth and striated muscle) there is a substance whose decomposition should occur just at the beginning of contraction, should be accompanied by a liberation of acid equivalents, the more the greater the *p*H, whose resynthesis must be very rapid and practically unaffected by monoiodoacetic acid: this substance is adenyl pyrophosphoric acid, discovered by Karl Lohmann (Lohmann, 1928, 1930, 1931, 1932, 1934 *a*, *b*; Embden & Zimmermann, 1927; Embden, Hefter & Lehnartz, 1930]. There are consequently certain reasons for supposing that variation (*b*) represents the transformation of adenyl pyrophosphoric acid to the diphosphoric acid or to adenylic acid. On the other hand, we know nothing at all concerning the alkaline variation (*a*).

Such are the phenomena which we have been able to detect at the surface of smooth muscle during activity. It is obvious that the changes in reaction which are recorded at the surface of the muscle occur later than the events taking place within the muscle fibre itself. The errors contributed by the glass electrode, the electrometer and the galvanometer are not very important in comparison with the slowness of smooth muscle contraction. But the lag is due principally to the fact that there cannot be an instantaneous equilibrium between the CO_2 concentration of the muscle fibre and that of the film of Ringer adjacent to the aponeurosis. We have no definite notions as to the magnitude of this delay due to diffusion time. We know only that the rapid alterations in reaction are necessarily modified more than the slower ones, and that consequently in our records phases (*a*) and (*b*) are more distorted than variation (*c*), and still more than (*d*). In view of these considerations we suggest that variation (*a*), which occurs so early in our records, may well represent an alkaline *pre-con-*

tractile phenomenon and that the process responsible for variation (*b*) occurs probably quite early in the contraction.

It is probable that phase (*c*) (phosphagen) in our records is in very close chronological relation to the muscle's activity. If this is true for (*c*), it holds *a fortiori* for (*d*) which corresponds to a still slower phenomenon.

B. *Quantitative results*. It is also self-evident that the non-instantaneous character of the CO_2 equilibrium makes it difficult to evaluate quantitatively the changes in reaction when these are rapid and transitory.

Variation (*a*), the most rapid of the four phases, is an alkalization which corresponds to a chemical process almost certainly much more important than the records disclose. On those records in which phase (*a*) stands out with sufficient clearness to enable a measurement of its amplitude without too great an error (this often occurs in fatigued muscles) we have found, with an initial *p*H between 7·15 and 7·40, 0·8, 1·4, 0·8 and 0·85 c.mm. of CO_2 were absorbed per gram of muscle.

Is variation (*b*) slow enough to be undistorted? The only records where the amplitude of this phase can be calculated with any precision are those where phase (*c*) is as small as possible, that is to say, those taken with very alkaline initial *p*H (7·20–7·40). It is under these conditions, moreover, that the amplitude of (*b*) is greatest. We have found diverse values, depending upon the muscles, and the tension developed, with a maximum of 5·5 c.mm. CO_2 liberated per gram of muscle (*p*H 7·22, 2 sec. stimulus, 210 g. tension developed, ring of stomach 17·8 mm. long and 9 mm. wide). The average is 3 c.mm. of CO_2 at *p*H 7·20. Attributing this acidity to the hydrolysis of adenyl pyrophosphoric acid, and taking into consideration the curve of electrotitration of this substance, these 3 c.mm. would correspond to a dephosphorylation of 0·00945 mg. of adenyl pyrophosphoric acid (H_3PO_4) $\left(\frac{\text{mg. } H_3PO_4}{\text{c.c. } CO_2} = 3{\cdot}15 \text{ at } p\text{H } 7{\cdot}20\right)$.

The (*c*) phase is generally very difficult to measure, because it evolves at a moment when phase (*b*) has not yet terminated and where phase (*d*) begins. The best measurements we have been able to make were on records of muscle poisoned to some degree by monoiodoacetic acid (in which consequently phase (*d*) was considerably reduced). On such records, at *p*H 7·20, we found for (*c*) from 0·5 to 2 c.mm. CO_2 absorbed per gram of muscle with an average of 1 c.mm., which corresponds to 0·0128 mg. of phosphagen (H_3PO_4).

Phase (*d*) is measured by the difference between the muscle *p*H before contraction and that found several minutes later, when the measurement is again stable. This method of measuring phase (*d*) is not quite exact since there is very likely an alkaline residue which arises from an incomplete resynthesis of phosphagen under anaerobic conditions. But inasmuch as this phase (*d*) is large in comparison with this residue, the error is practically negligible. In fact, phase (*c*) has an average maximum of 1 c.mm. (*p*H 7·20), phase (*d*) 4 to 8 c.mm. (average 6 c.mm.). Since the residue from (*c*) attains at the most only half of its maximum amplitude, the error cannot exceed 0·5 c.mm. CO_2 in the estimation of phase (*d*).

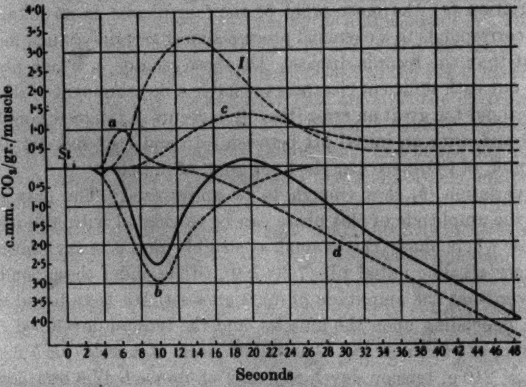

Fig. 9. Continuous line, variation in CO_2 concentration of the Ringer film covering the smooth muscle during a contraction, such as results from the *p*H curve experimentally determined ($\log CO_2 = 4{\cdot}56 - p\text{H}$). (*a*), (*b*), (*c*), (*d*), component phases, whose resultant is given by the continuous line. *I*, isometric contraction. *St*, stimulation.

Recapitulation of the results. Changes at the surface of smooth muscle during contraction. The average results of the clearest records are used. Initial *p*H is 7·20 (contraction following a stimulus of 1 to 2 sec.):

Phase (*a*), alkaline, probably pre-contractile, less than 1 c.mm. CO_2 per gram muscle; this value is very likely higher in the muscle fibre itself. The maximum of this phase is attained at the outset of the period of increasing tension. It appears to be unaffected by poisoning with monoiodoacetic acid.

Phase (*b*), acid, maximum 5 c.mm. CO_2 per gram of muscle at the surface of the tissue (average 3 c.mm.); in the muscle fibre this figure may be exceeded. This phase begins before the contraction and its maximum is reached toward the middle of the period of increasing tension. In a given muscle, it is proportional to the tension developed. It is smaller if the initial *p*H is smaller. This phase is probably due to the hydrolysis of adenyl pyrophosphoric acid. It would correspond to the dephosphorylation of about 0·00945 mg. of adenyl pyrophosphoric acid (H_3PO_4). This phase appears to be unaltered by monoiodoacetic acid poisoning.

Phase (*c*), alkaline, 0·5–2 c.mm. CO_2 per gram muscle (average 1 c.mm. CO_2). This phase begins during the period of increasing tension. Its maximum is reached at the beginning of relaxation; it is maintained at this maximum in monoiodoacetic acid poisoning. Its amplitude increases with decreasing *p*H. It corresponds to the hydrolysis and synthesis of creatine phosphoric acid, of which the average quantity decomposed is 0·0128 mg. (H_3PO_4).

Phase (*d*), acid, from 4 to 8 c.mm. CO_2 per gram muscle (average 6 c.mm. CO_2). Its amplitude is independent of the initial *p*H of the muscle. It begins during contraction; its maximum is reached several minutes later. This phase disappears in poisoned muscles. It corresponds to the formation of 0·024 mg. lactic acid per gram muscle.

The approximate evolution, temporally, of these four phases, at the surface of the muscle, is represented in Fig. 9.

Results of observations on striated muscle

A review of present knowledge of alterations in *p*H in relation to muscular contraction will be found in Dubuisson [1937*b*, *d*]. We have previously shown that in excitation of a muscle, normal or poisoned, there occurs during the 20–100 initial contractions or during the first two to five 2 sec. tetani, a progressive alkalization of the muscle. The maximum of this alkalization is the same as would occur in non-formation of lactic acid (when the muscle is poisoned by monoiodoacetic acid, mono- and dihexosephosphoric acids are formed) and this alkalization is greater with decreasing initial *p*H. Once this maximum of alkalization is reached, the following contractions produce a progressive acidification of the normal muscle (an acidification which does not depend upon the initial *p*H of the muscle), whereas the muscle poisoned by monoiodoacetic acid terminates its activity in contracture, without acidification.

We have shown that in a normal muscle, or a monoiodoacetic acid poisoned one, there is an excellent correlation between the changes in *p*H and the chemical proportions of phosphagen hydrolysed, of esters, and of lactic acid ultimately formed.

In one of these investigations [Dubuisson, 1937*d*, Fig. 2] *during contraction* there was, in addition to each tetanus, an *acidification* of the muscle. At that time I was not certain whether this variation was an artefact or not. Actually, there is no longer any doubt that this figure was free from artefacts. With our improved technic, the acidification of muscle during a 1–2 sec. tetanus appears in each of our records (Figs. 10, 11). The systematic study of this acidification, in muscles placed initially in *p*H's varying from 6·10 to 7·40, enables us to establish that this acidification is greater when the muscle is initially more alkaline (like phase (*b*) of smooth muscle). On the other hand, this acidification decreases with fatigue. In a 5 sec. tetanus of a muscle already fatigued, it is observed only during the first or the first 2 sec. of the contraction; then the muscle becomes, on the contrary, alkaline (Fig. 11). This alkalization increases until the end of the tetanus, and starting from the end of this 5 sec. tetanus the *p*H returns to about what was effectively its initial value. This shows the predominance of an alkaline reaction (phase (*a*) of smooth muscle?) over the acid reaction in a fatigued tissue during contraction. After the contraction, appears again an alkalization, slower and more progressive, which chemical analyses relate to phosphagen hydrolysis. Here, then, we find as in smooth muscle, before the phosphagen

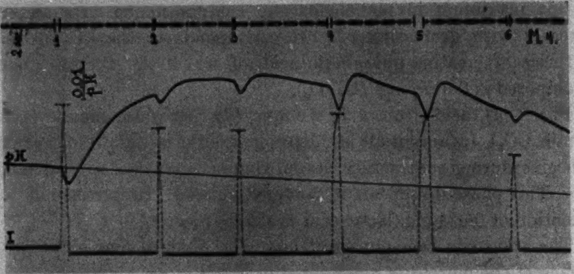

Fig. 10. Frog gastrocnemius. Normal fresh muscle. I, isometric contraction. *p*H, variations in *p*H (acid below, alkaline above). The muscle becomes acid during the contraction (cf. phase (*b*) of smooth muscle) and *this acidity is proportional to the surface of the mechanical record*. The alkalization which ensues after each contraction corresponds to the hydrolysis of phosphagen and the acidification which follows, in the four last tetani, from lactic acid (whose acidity here surpasses the alkalinity owing to phosphagen decomposition).

Initial *p*H, 7·12. Temp. 20° C.

phase, an alkaline and an acid phase, and the last one with the same characteristics as the acid phase (*b*) which we described in the course of the ascending portion of the smooth muscle's mechanical record. In both cases this acidity increases with initial *p*H and decreases with fatigue.

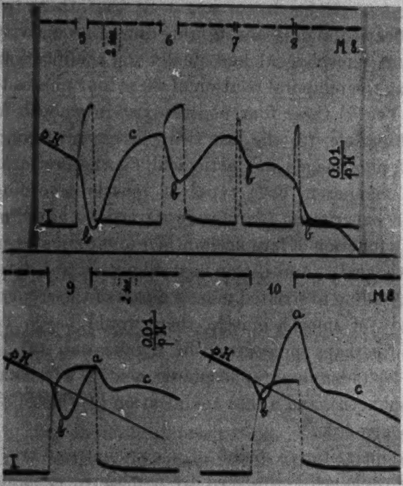

Fig. 11. Frog gastrocnemius. Normal muscle. Changes in *p*H (acid toward the bottom, alkaline toward the top) during the last six tetani of a series of ten. It can be seen that the muscle invariably starts by becoming acid (phase (*b*) of smooth muscle). But, if the contraction is prolonged (long tetani, nos. 6, 9 and 10) and the muscle is already fatigued by earlier contractions, this acidification is followed, during the contraction, by an alkalization, immediately reversible after the relaxation. This shows the predominance of an alkaline phase (phase (*a*) of smooth muscle?) over the acid phase (phase (*b*) of smooth muscle) in prolonged tetanic contractions of fatigued striated muscles.

During long tetani of fatigued muscles, the alkalization always finally dominates the acidification (Fig. 11). The interpretation of these complex facts, which so long appeared to us incomprehensible, appears to me to be now given in the light of what we have found in smooth muscle; there are probably, in both cases, shortly after stimulation two reactions of different sign, one alkaline, not very sensitive to fatigue, the other acid, affected by fatigue.

The distortions introduced by the non-instantaneousness of the CO_2 equilibrium, by the glass electrode, the electrometer and the galvanometer evidently have a considerably greater importance for striated than for smooth muscle, whose activity is 50–100 times slower. Moreover, if one were content to consider only the results on striated muscle, the only conclusion that could be drawn might be that there occurs a simultaneous acidification and alkalization(?) during its activity, followed by an alkalization (phosphagen) and finally an acidification (lactic acid) without stating the temporal relation of these four phases to the muscle's contraction. Yet (1) these four phases exist in smooth muscle as well as in striated muscle, (2) the last two phases correspond to processes linked up with phosphagen and lactic acid, (3) the first acid phase varies in analogous manner in both types of muscle according to the conditions of the experiment (amplitude variations as a function of the initial *p*H, as a function of fatigue and their invariance when treated with monoiodoacetic acid). And if this now allows us to believe that we are justified in extending to striated muscle a part of those conclusions drawn from the study of smooth muscle, there would be, in both instances, a very early, perhaps precontractile, alkalization (*a*), a contraction acidification (*b*) (adenyl pyrophosphoric acid?), a relaxation alkalization (*c*) (phosphagen), and a late acidification (*d*) (lactic acid), predominantly post-contractile.

From the quantitative point of view, matters are different: the amounts of phosphagen hydrolysed are much greater in striated than in smooth muscle. To be convinced of this, it is necessary only to compare the *p*H variations, as well as the results from chemical determinations. As for the initial phases in striated muscle, which by an analogy with those of smooth muscle we will call (*a*) and (*b*), we cannot at present give any quantitative estimation, the CO_2 diffusion time being certainly greater than the speed with which these phases are evolved at the surface of striated muscle. For the present we must refrain from further considerations of striated muscle. We will return soon to this problem.

DISCUSSION

Amongst the diverse methods used hitherto in the study of those processes which appear in the course of muscle contraction (action potentials, volume changes, light transmission, birefringence, chemical constitution, heat production, impedance) few are adapted to a quantitative analysis, and often they depend upon reactions whose temporal relations are imperfectly known. The technique of recording *p*H variations in the course of the contraction fills up part of this gap, since it enables one not only to detect a certain number of muscular reactions but also to follow them quantitatively and to locate them chronologically. From this point of view the study of heat production has already given us valuable information, and doubtless a particular interest attaches to the comparison of the results of these two methods of investigation, and to see whether the chemical processes disclosed by pH variations can explain the evolution of the heat production curve.

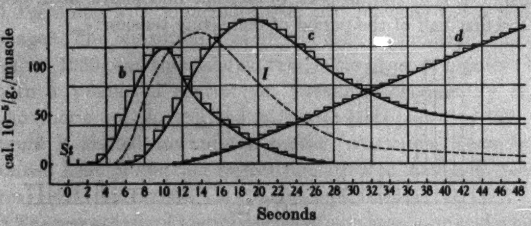

Fig. 12. In this diagram, the elementary phases (*b*) (adenyl pyrophosphoric acid), (*c*) (creatine phosphoric acid) and (*d*) (lactic acid) of Fig. 11, are represented in cal. × 10^{-5} per gram muscle. The blocks represent successive increments and decrements. *I*, isometric contraction.

Knowing nothing about heat production, how could the qualitative and quantitative determinations made in this work enable us to depict it? Let us neglect phase (*a*) which we cannot relate logically to anything already known and limit our calculations consequently to phases (*b*), (*c*) and (*d*). From the averages of our determinations on smooth muscle we can reconstruct Fig. 11 by replacing c.mm. CO_2 by the corresponding quantities of adenyl pyrophosphoric acid, phosphagen and lactic acid respectively We have:

(*b*) = 0·00945 mg. adenyl pyrophosphoric acid (H_3PO_4).
(*c*) = 0·0128 mg. phosphagen (H_3PO_4).
(*d*) = 0·024 mg. lactic acid.

Knowing the heats of reaction: adenyl pyrophosphoric acid = 12,000 cal./mol. phosphoric acid, phosphagen = 11,000 cal./mol., formation of lactic acid = 16,000 cal./mol. (to which is to be added about 8000 cal. for heat of neutralization), we find:

$$\left.\begin{array}{l}(b)\ 116\ \text{cal.} \times 10^{-5}\\(c)\ 145\ \text{cal.} \times 10^{-5}\\(d)\ 640\ \text{cal.} \times 10^{-5}\end{array}\right\}\ \text{per gram of muscle (Fig. 12).}$$

The velocity of production of these quantities of heat can be determined by measuring successive increments in the curves of Fig. 12. By summing algebraically, for 2 sec. intervals, the simultaneous increases and decreases, a curve representing the resultant of heat production in the muscle is obtained (Fig. 13). The record may be compared with known curves of heat production. Fig. 13 shows that there can be distinguished during an anaerobic contraction of smooth muscle:

(1) an important positive heat, due to the decomposition of adenyl pyrophosphoric acid and, in part, to creatine phosphoric acid, occurring during the first half of the period of increasing tension;

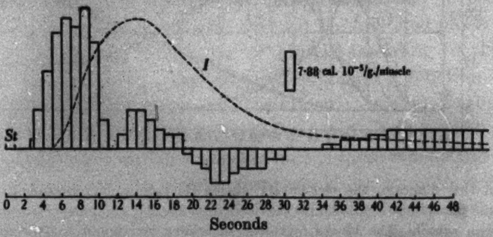

Fig. 13. Rate of heat production during the contraction of smooth muscle, such as is obtained from calculations based on *p*H changes. *I*, isometric contraction.

(2) an almost isothermal period, slightly ahead of the maximum tension, and which results from the endothermic resynthesis of adenyl pyrophosphoric acid and the exothermic hydrolysis of phosphagen;

(3) a second heat production, less important than the first, which corresponds to maximum tension and to the beginning of muscle relaxation and which is largely due to the exothermic hydrolysis of phosphagen;

(4) a period of negative heat which results both from endothermic resynthesis of creatine phosphoric acid and from exothermic formation of lactic acid;

(5) a period of positive heat, constant for many minutes, and which represents the delayed exothermic formation of lactic acid.

As can be seen, there is a striking analogy between this diagram and the heat production curves such as have been established by Hill, Hartree and their collaborators, who have found, under anaerobic conditions, a positive heat of contraction, a positive heat of maintenance and relaxation, a negative post-contractile heat, then a more or less pronounced positive prolonged heat. Without being perfect, the analogy is too striking to be due only to a coincidence. We think that it is these chemical processes which also account for alterations in muscle *p*H that underlie the production of heat in muscle. Our knowledge of the relations between these three chemical processes also enables us to explain the variations in form which have been given by heat production curves recorded either during fatigue or poisoning by monoiodoacetic acid or when the stimulation is tetanic. Several examples may be cited:

(*a*) Fatigue diminishes the speed of resynthesis of adenyl pyrophosphoric acid [Lohmann, 1928]. The isothermal period (2) will then tend to become positive [observed by Hartree, 1931, 1933].

(*b*) Poisoning with monoiodoacetic acid prevents the resynthesis of phosphagen. The relaxation heat should then be prolonged [ascertained by Fischer, 1930] and there should then be no phase of negative heat (?).

(*c*) In a prolonged contraction (tetanus) the quantity of adenyl pyrophosphoric acid hydrolysed is greater [this paper, p. 469] and the maximum is shifted toward the tension maximum. There is then a maintained heat production from stimulation to maximum tension [cf. Hartree, 1933, contraction heat, maintenance heat].

(*d*) The resynthesis of hydrolysed creatine phosphoric acid is unimportant in an initial tetanus, but is accomplished more completely in succeeding tetani [Meyerhof & Möhle, 1933 *a*, *b*]. The negative post-contractile heat would then be slightly developed in the first tetanus, more so in the later ones [cf. Hartree, 1932, 1933].

SUMMARY

In the course of the isometric contraction of smooth muscle, four successive changes in reaction are produced, designated by the letters (*a*), (*b*), (*c*) and (*d*).

(*a*) An alkaline phase, beginning before the contraction, maximum at the outset of the period of increasing tension. Corresponds to an unknown chemical process.

(*b*) An acid phase. Begins before contraction, maximum toward the middle of the period of rising tension. It is proportional to the tension developed. It is probably an expression of the decomposition and resynthesis of adenyl pyrophosphoric acid.

(*c*) An alkaline phase. Begins during the period of rising tension, maximum at the outset of relaxation. It represents the hydrolysis and synthesis of creatine phosphoric acid.

(*d*) An acid phase. Begins during contraction, maximum several minutes later. It is an expression of the formation of lactic acid.

The (*b*), (*c*) and (*d*) changes in reaction have been calculated quantitatively for a contraction resulting from a 1 to 2 sec. stimulus, and the

equivalents of adenyl pyrophosphoric acid, phosphagen and lactic acid expressed in mg. of substance decomposed per gram of muscle (for about 200 g. tension) or in calories per gram of muscle. In this way we have been able to outline a *calculated* heat production on these chemical bases, and we have found a good agreement between the diagram obtained and the curves of heat production measured directly by other investigators.

The four changes (*a*), (*b*), (*c*), (*d*) in hydrogen-ion concentration are also found in striated muscle, but they are more difficult to study in this tissue, owing to the rapidity of the contraction.

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EXPERIMENTAL INVESTIGATIONS OF THE RESERVOIR BLOOD OF THE CAT SPLEEN: WITH SPECIAL REFERENCE TO HAEMOLYSIS AND THE SEDIMENTATION REACTION

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One of the questions most discussed in connexion with the physiology of the spleen has been that of the part played by that organ in the destruction of red blood corpuscles.

The evidence for the supposed phagocytosis of erythrocytes in the spleen has been surveyed by Rous & Robertson [1917], Rous [1923], Krumbhaar [1926], Lubarsch [1927], Brinkman [1928] and Lauda [1933, 1937]; Knisely [1936 *b*, *c*] never observed phagocytosis in the living spleen, but, on the other hand, considerable phagocytosis in the same organ a few minutes after death.

According to Rous & Robertson [1917], Lauda [1933, 1937], Doan & Sabin [1926], Albendin [1936], and others, a continual extracellular, mechanical fragmentation of red blood corpuscles takes place in the circulating blood. It is presumed that the fragments are captured and ingested by the reticulo-endothelium, particularly in the spleen [Addison, 1920; Lauda, 1933]. Rous [1923], however, denies that fragmentation occurs to any extent, or that any such fragments accumulate appreciably in the spleen.

It is not known with certainty if extracellular haemolysis to any considerable extent takes place in the normal organism [Brinkman, 1928]. Apart from observations on the venous blood of the spleen, Fåhraeus' demonstration in 1924 of small quantities of "colourless globules" (farblose Blutkugeln) in the normal circulating blood seems so far to be the only direct evidence of a normal extracellular haemolysis. Eppinger [1913, 1920] has, on vague grounds [cf. Lauda, 1933, 1937], presumed that the red blood corpuscles, when passing through the spleen, are prepared for haemolysis which, in a second phase, is completed in the liver. A number of writers, however, have found that the osmotic resistance of the red blood corpuscles becomes reduced when they pass through the spleen [Eppinger, 1920; Bolt & Heeres, 1922; Barcroft, 1926*a*; Orahovats, 1926; Deleonardi & Paolazzi, 1936; Gordon & Kleinberg, 1937; and others]. This has been considered as a change preliminary to haemolysis [Eppinger, 1920]. Lauda [1933, 1937], on the other hand, rightly points out that reduced osmotic resistance does not necessarily imply a change in the red blood corpuscles that facilitates their intravital haemolysis. All previous positive results of attempts to extract haemolysin from spleen parenchyma [Gilbert, Chabrol & Benard, 1912] seem recently to have been more or less disproved [Widal, Abrami & Brulé, 1912; and others].

Gray observed in 1854 that serum taken from the splenic venous blood of horses immediately after death regularly showed a red-brown colour after the evaporation of the blood, in contradistinction to serum from arterial or other venous blood. The cause may possibly have been due to free haemoglobin. Similar observations of free haemoglobin in the venous blood of the spleen are mentioned by Rous [1923] as well as by Krumbhaar [1926] and Brinkman [1928] (without any references to the literature). On the basis of his own investigations, however, Krumbhaar writes [1926, p. 175] "the slight and inconstant differences observed" (in osmotic resistance and haemoglobinaemia) "were well within the limits of error inherent in the relatively crude methods of blood examination (or in some cases to technical errors)". Brinkman states [1928] that most workers in this field [also cf. Eppinger, 1920] have been unable to find the alleged haemoglobinaemia in the splenic vein.

One reason for the great variability in the statements regarding haemoglobinaemia in the blood of the spleen vein may perhaps be due to lack of consideration to the reservoir function of the spleen. Bergenhem & Fåhraeus [1936] have found that the blood of man, and all animals examined, changes in a regular manner when kept stagnant at body temperature. The change, which is sometimes quite pronounced after only a few hours, is apparent in the transition of the normal form of the erythrocytes into a more or less spherical one, in a reduction of their rouleaux formation and a consequent decrease of their sedimentation speed. This change of the erythrocytes, considered to be a preliminary stage of haemolysis, is at least partly effected by primary changes in serum (or plasma) which are due to lysolecithin formation. The most interesting result in these investigations is that this lysolecithin formation in the plasma does not manifest itself if the blood is kept flowing during the experiment. On the basis of these observations and on finding that the venous blood of the spleen is stabilized, i.e. has a lower sedimentation reaction, as compared to the peripheral circulating blood [Frenckell & Wyssotsky, 1927; Gawrilow, 1928; Berglund, 1930], Bergenhem & Fåhraeus [1936] draw the conclusion that the fact that the blood comes to rest in the spleen during the reservoir phase involves lysolecithin formation, viz. a change predisposing towards haemolysis.

According to investigations by Bergenhem, continued in this institute and soon to be published, the lysolecithin formation in the plasma of the stagnant blood depends upon the separation of the erythrocytes from the plasma which is brought about *in vitro* by sedimentation of the erythrocytes, and in the spleen by a mechanism described by Knisely [1936*b*, *c*] (see below).

METHOD

The experiments have been carried out on cats, chiefly because the cat's spleen has a high reservoir function [cf. Barcroft, 1926*a*, *b*, 1930; Asher, 1937; von Herrath, 1935; Knisely, 1936*b*; Guillery, 1938], and also because cat blood usually shows a sufficient sedimentation reaction to make it possible to study the stabilization, i.e. reduction of the sedimentation speed. As a rule the cats were fed as usual before the start of the experiment. In order to get the greatest possible depot in the spleen, the animals were put under chloralose narcosis (0·05–0·07 g. chloralose per kg. of body weight; for the advantages of the method, see Barcroft & Florey [1928] and Goreczky & Ludány [1937]) and left at room temperature for varying periods, as a rule between 3 and 4½ hr. Thereupon, heparin (0·01 g. heparin "Vitrum" per kg. of body weight) has, as a rule, been slowly injected intravenously, dissolved in 5 c.c. of physiological NaCl solution at body temperature.[1] Five minutes after the heparin injection the abdomen was opened, the ligamentum hepatoduodenale with vena porta quickly nipped with Péan forceps, the stagnated spleen vein ligated and the forceps thereafter taken away. A cannula was inserted into the spleen vein and the blood collected in a series of test tubes. Coincidently the spleen was made to contract by touching it with the finger. Finally, blood for comparison was taken from carotis communis, and in several tests also from vena cava inferior and vena jugularis externa.

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[1] According to Jorpes [1936], 0·00075 g./kg. body weight should be enough to prevent the coagulation of the circulating blood; as, however, a more or less effective isolation of the spleen depot from the circulating blood must obviously be reckoned with [Barcroft, 1926*a*; Henning, 1927; and others] this comparatively large quantity has been applied.

The quantities of the blood samples thus obtained were determined and the blood examined for sedimentation reaction, for the volume percentage of red blood corpuscles and for the haemolysis colour of the plasma. After centrifugation of the blood samples in haematocrit tubes (3 mm. inner diameter × 50 mm. long), the volume of the red blood corpuscles was determined with a measuring microscope. The degree of haemoglobinaemia in the free plasma layer was measured colorimetrically by comparing it with a standard scale of haemolysed blood.

The first experiments showed that the degree of haemoglobinaemia in the spleen blood increased with time. The interval between blood-sampling and haemoglobin determination must consequently be considered. In one group of experiments the blood was collected in cooled tubes, examined as soon as possible, and kept for certain periods of time at body temperature and then re-examined. In other experiments the blood was collected in tubes at body temperature, and finally in a third group of experiments in tubes at room temperature.

In all, the experiments included nineteen animals.

RESULTS

Table I is a summary of all the material. In the main group (nos. 1–10) the spleen was found dilated, dark red and with distended capsule when the abdomen was opened. (An exception to this is, for reasons unknown, no. 7, see Table I.) During the drawing of the blood from the spleen vein the spleen contracted as a rule to one-quarter of its original size, turned light red, became firmer in consistency and slightly granulated. The amount of blood varied for the originally big organs between 21 and 41 c.c. and averaged about 30 c.c. From a small spleen (no. 7) no more than 12 c.c. could be drawn. As can be seen in Table I, the volume of splenic venous blood obtained is proportional to the original size of the organ. In these experiments it was interesting to see how the flow of blood from the spleen vein always decreased in proportion to the contraction of the organ.

The concentration of erythrocytes in the venous blood obtained by the contraction of big spleens shows regular variations. The first 3 or 4 c.c. have usually shown nearly the same haematocrit value as the controls (vena cava inf., vena jugul. ext. and arteria carotis). The value has then increased rapidly during the contraction of the organ, reached a maximum when about half the volume has been drawn and then slowly decreased to the same value as those of the controls (cf. Fig. 1, which shows a typical

TABLE I

					Venous blood from spleen					Controls: max. haemoglobinaemia after the same length of time			
						Erythrocytes		Maximum haemoglobinaemia†					
Group	Exp. no.	Weight of animal kg.	Duration of narcosis hrs.	Size of spleen when abdomen opened	Vol. c.c.	Max. conc. %	Surplus in c.c.*	Immediately after drawing	After time indicated	Vena cava inf.	Vena jugul. ext.	Art. carotis	Treatment of samples
I	1	4·6	4·3	Big	43	58	9·8	1·5	60 m.: 6·5	1·1	—	0·7	Collecting in cooled tubes, later at 38–39° C.
	2	3·5	4·3	Medium	18	55	2·3	2·2	30 m.: 6·4	—	—	0·5	
	3	3·4	4·5	Very big	31	71	9·2	1·3	15 m.: 3·0 45 m.: 3·8	—	—	— 0·3	
	4	4·0	4·3	Huge	41	69	9·7	0·9	15 m.: 4·8 45 m.: 9·3	—	—	— 0·6	
	5	2·3	3·8	Big	40	64	10·0	—	20 m.: 1·0	—	—	0	
	6	4·1	3·8	Big	24	71	11·8	—	30 m.: 0·4 60 m.: 0·7	— 0	— —	— 0	Collecting direct in tubes at temp. 38–39° C.
	7	3·1	4·3	Small	12	63	3·3	—	6 m.: 0·4 42 m.: 0·5	— 0·4	—	— 0·4	
	8	3·0	3·5	Medium	—	56	—	0·6	25 m.: 1·1	—	0	0	
	9	3·1	3·5	Big	21	62	5·9	—	51 m.: 2·2	2·8	0·5	0·5	
	10	4·1	4·0	Big	28	67	—	—	60 m.: 4·2	0·4	—	0·4	At room temperature only
II	11	2·9	5·5	Big	—	74	—		1·0–2·1	0·3	—	0·3	
	12	3·5	6·0	Huge 48 g.	—	70	—		>5·0	—	—	—	
	13	2·4	6·5	Small 16 g.	—	42	—		0·3	—	—	0	
	14	3·3	7·0	Medium 22 g.	—	59	—		0·6	—	—	0	
	15	3·5	6·0	Huge 48 g.	—	—	—		>5·0	—	—	0	
III	16	3·5	5·5	Small	—	—	—		—	—	—	—	
	17	2·5	5·0	Small	—	—	—		0	—	—	—	
IV	18	2·9	3·3	Big	—	63	—	0	0	—	—	0	As nos. 1–5 above
	19	3·8	4·3	Big	—	55	—	—	15 m.: 0·5	—	—	0	

Group I: The normal method described. Group II: Different methods, see text. Group III: The animals under narcosis in a high exterior temperature; otherwise as in Group I. Group IV: The animals were starved; otherwise as in Group I.

* Means the difference between the volume of erythrocytes in the splenic venous blood collected and the volume of erythrocytes in the same volume of carotid blood.

† The haemoglobinaemia in the experiments nos. 1–10 is expressed as the number of destroyed erythrocytes per thousand total erythrocytes; in the experiments nos. 11–19 in number of c.c. of destroyed erythrocytes per 1000 c.c. blood.

curve, *a*). The average maximum concentration of erythrocytes in the blood of the *spleen vein* was 64 %; the averages for the haematocrit values of the controls were as follows: vena cava inf. 44 %, vena jugul. ext. 41 %, arteria carotis 39 %.

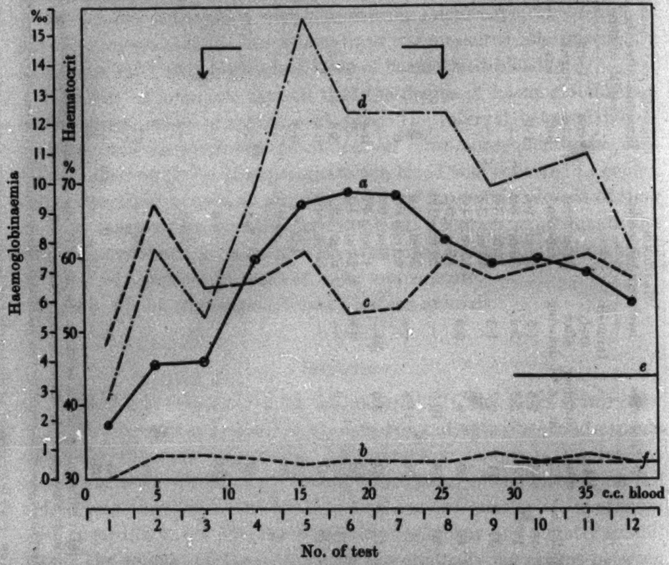

Fig. 1. Reservoir blood of the spleen: haematocrit values and haemoglobinaemia. Exp. 4 (cf. Table I). Curve *a* shows the volume percentage of erythrocytes in the blood from vena lienalis, collected during the contraction of the spleen; chief contraction of the spleen between ⬐ and ↴. Line *e* marks volume percentage of erythrocytes in the carotid blood. Curve *b* shows the haemoglobinaemia found in blood just drawn from vena lienalis, expressed as destroyed blood corpuscles per thousand total (the scale farthest left); corresponding haemoglobinaemia in the carotid blood: 0. Curve *c* shows the relative amount of destroyed blood corpuscles in the same samples 45 min. after drawing; line *f* marks the corresponding value in the carotid blood. Curve *d* shows the haemoglobin concentration, read off direct, in the plasma of the venous blood of the spleen 45 min. after drawing (curve *c* has been computed on the basis of *a* and *d*).

These results agree very well with previous investigations [Barcroft, 1926*a*, *b*, 1930; Barcroft & Stephens, 1927, 1928; Barcroft & Poole, 1927–8; Barcroft & Florey, 1928; Scheunert & Krzywanek 1926;

Lauda, 1933; and others]. The connexion between the volume of blood and the size of the spleen, together with the haematocrit curve, certainly show that reservoir blood has been obtained. As there was no coagulation, the blood in the spleen vein must have contained heparin and thus a certain amount of "passage blood". The quantity of the latter cannot be ascertained, but from the high maximum of the haematocrit value (about the same as Cruickshank [1926] found in the spleen vein after ligating the artery) as well as from previous investigations into the organ's capacity to choke the arterial stream during contraction [Henning, 1927; Lauda, 1933; Guillery & Petersen, 1935; Knisely, 1936*a*, *b*] it is justifiable to conclude that only a comparatively small portion of circulating blood was intermingled.

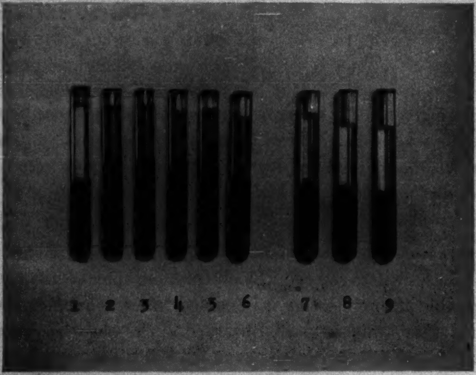

Fig. 2. Haematocrit tubes from Exp. 9; centrifuged 51 min. after the blood was drawn. Tubes 1–6 venous blood from the spleen, tube 7 sample from vena cava inferior, tube 8 from vena jugularia externa, and tube 9 from arteria carotis.

Sedimentation reaction (*S.R.*)

The sedimentation reaction was performed in seven animals all of which emptied an obviously distended spleen (nos. 1, 2, 3, 4, 8, 9 and 10 in Table I). The reservoir blood drawn in all cases showed a very low S.R. As is known, the rate of sedimentation of the erythrocytes is essentially determined by two factors, i.e. by the aggregation tendency of the erythrocytes and by their percentage quantity [Fåhraeus, 1921, 1929]. With regard to this, samples with equal concentrations of erythrocytes have been compared in four of the aforementioned experiments. In order to obtain such samples, 1 c.c. of heavily centrifuged blood corpuscles was mixed with 2 c.c. of the plasma from which they had just been centrifuged. Fundamentally, the results of all the tests agree (see Table II in which two are given): *The reservoir blood of the spleen shows, in relation to the peripheral venous and arterial blood, a very low S.R., a fact which is evidently partly due to a reduced aggregation tendency in the reservoir blood's erythrocytes and not only to the high erythrocyte concentration.*

TABLE II. Sedimentation reaction (S.R.) with a constant concentration of erythrocytes (1 part of blood corpuscles + 2 parts of plasma)

No.	S.R. observed after min.	Vena lienalis mm.	Vena jugul. ext. mm.	Art. carotis mm.
8	30	9	53	46
	60	28	91	80
	120	72	122	121
9	30	2·5	9	33
	60	7	31	67
	120	17	71	116

Haemoglobinaemia

In those cases where reservoir blood from big spleens was examined as soon as possible after collecting it in cooled tubes, a haemolysis colour—faint or moderate—was regularly observed in the plasma; controls (from vena cava inf. and arteria carotis) examined in the same way showed no haemoglobinaemia. As a rule, there was only faint or practically no haemolysis colour in the first cubic centimetres of plasma. In the subsequent ones an increase was noted which usually reached a maximum in the same samples as those showing haematocrit maximum.

The reservoir blood, having been cooled while being shed and then kept for different periods (15–60 min.) at 38–39° C., regularly showed an obvious and often very strong increase of haemoglobinaemia. In the controls under the same conditions the haemoglobinaemia was entirely absent or scarcely measurable. When found the haemoglobinaemia of the circulating blood was always considerably less than that of the spleen blood, even if the values of haemoglobin concentration of the plasma were corrected for the fact that the spleen blood was much richer in corpuscles and much poorer in plasma than the circulating blood. This is plainly shown in Table I (nos. 1–5) where the values in the separate tests have been given. The increase of the haemoglobinaemia and its distribution in the series of samples is also made clear by Figs. 1 and 3 for the typical experiment no. 4.

Fundamentally, the same reaction was shown by reservoir blood from big spleens which had not been cooled on drawing but kept at body temperature (or at 38–39° C., nos. 6, 8 and 9), or at room temperature (no. 10). However, the absolute value of the haemoglobinaemia in this group of experiments, in most cases, turned out lower than in the previous group (see Table I) which seemed to justify the belief that cooling favoured the increase of haemoglobinaemia. This is probably not the case, however, for in a parallel series of samples where one was cooled and then warmed—the other was only kept warm—no definite effect of cooling could be observed (method applied in tests of experiments nos. 7, 8 and 9). As regards the controls it should be added that in one case the blood from vena cava inf. after 51 min. at 38–39° C. showed approximately the same haemoglobinaemia as did the reservoir blood of the spleen (no. 9), to which I shall return later; in all the other tests the reservoir blood of the spleen differs strikingly from the controls (see Table I).

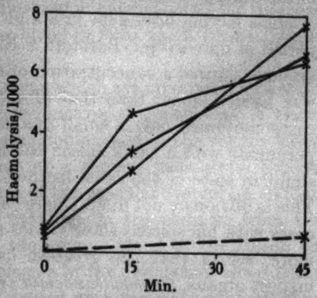

Fig. 3. From experiment no. 4. Full lines: haemolysis of three different samples of venous blood from the spleen, expressed as the number of destroyed erythrocytes per thousand total. Broken line: haemolysis of the carotid blood.

The method was varied as follows in five cases: in one animal (no. 11) no heparin injection was given. All the hilus vessels of the spleen were nipped with Péan forceps during the contraction of the spleen and the drawing of the samples from the spleen vein. The blood was collected in tubes moistened with heparin in 0·9% NaCl solution. In three cases (nos. 12, 13, 14) the spleen—with Péan forceps nipping the spleen vessels—was extirpated and the venous blood drawn from the severed vein was collected in isotonic sodium citrate solution. In one case, finally, the spleen was removed and cut up over a funnel and the collected blood left to coagulate at room temperature, whereupon the serum spontaneously pressed-out was examined (no. 15). The results agree with those previously described (see Table I).

It is thus found that *spleen reservoir blood*, such as can be drawn from a cat which has lain still in a chloralose narcosis for 3½ hours or more, *shows a decreased aggregation of the erythrocytes and a marked haemoglobinaemia which increases spontaneously* in vitro.

In four experiments the spleen was found small when the abdomen was opened (nos. 7, 13, 16 and 17). In two cases the reason was unknown, since the animals (nos. 7 and 13) had received the same treatment as the other animals in the same groups and which had shown dilated spleens; in the other two cases (16 and 17) the contraction was probably due to the animals having been kept in a high outer temperature during the whole period of narcosis [cf. Barcroft & Elliott, 1936]. Moreover, as these four animals showed a concentration of erythrocytes in the spleen blood that was hardly higher than that of the controls, the spleen blood in these cases contained only a small quantity of depot blood.[1] In all these experiments with a small spleen the haemoglobinaemia proved to be very slight in spite of the same method being adopted as in the other tests. This is obviously not due to mere chance, for only two out of the thirteen cases with big spleens show values nearly as low (see Table I). Furthermore, seeing that the greatest haemoglobinaemia was found in the biggest organs, *haemoglobinaemia is, in all probability, connected with the reservoir function of the spleen.*

In every one of the above experiments the animals were fed 2–3 hr. before the narcosis began. In order to ascertain whether digestion influences the onset of haemoglobinaemia, two animals which had been starved 24 hr. (but had had access to water, nos. 18 and 19) were subject to the same examination as the main group nos. 1–10. In both experiments a big spleen contracted when the venous blood of the spleen was drawn, and the haematocrit curves were typical of reservoir blood. In one case no haemoglobinaemia was found in the depot blood, in the other a rather slight haemoglobinaemia was apparent after 15 min. at 38–39° C. (see Table I). Thus it seems possible that the onset of haemoglobinaemia in the reservoir blood is increased during digestion; no decisive conclusions can, however, be drawn from these few observations.

The osmotic resistance of the reservoir blood was examined in one case only (no. 4, Hamburger's method) immediately after the blood had been drawn. A reduced resistance was found as compared with that of carotid blood. (Depot blood: maximum resistance 0·46% NaCl, minimum resistance >0·62%; carotid blood: 0·44 and 0·56% respectively.)

As a subordinate finding it may be noted that the reservoir blood was always strikingly dark.

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[1] No. 7, however, is not quite clear, the venous blood of the spleen there showing a concentration of as much as 63% erythrocytes; the controls were also exceptionally rich in erythrocytes, viz. for vena cava inf. 59% and for arteria carotis 48%.

DISCUSSION

Of the results obtained, the haemoglobinaemia is of greatest interest. A distinction can be made between the faint haemoglobinaemia found in the venous blood of the spleen immediately after being drawn—which in the following I shall name primary haemoglobinaemia—and the increase of the haemoglobinaemia of the same blood *in vitro* which I have named secondary haemolysis.

What is the cause of these phenomena which are obviously connected in one way or other with the reservoir function of the spleen? *A priori* one might suppose that primary haemoglobinaemia in the venous blood of the spleen is a mere consequence of a general haemolytic process which may be as extensive in all parts of the vascular system [cf. Widal, *et al.* 1912], but which is not as manifest in the samples from the circulating blood as in those from the venous blood of the spleen on account of the haemoglobin in the plasma of the circulating blood being constantly resorbed. Nor can it be denied with certainty that primary haemoglobinaemia in the venous blood of the spleen is a consequence of the reservoir function of the spleen only in so far as haemoglobin is not so extensively resorbed from the plasma in the reservoir blood of the spleen as in the circulating blood. But this does not suffice to explain the more interesting result of this investigation, viz. the fact that haemoglobinaemia in the spleen plasma after it has been drawn increases incomparably more than it does in the circulating blood. This secondary haemolysis in the spleen's reservoir blood must be due to a particular influence to which the blood is subject when in the spleen and which does not affect the circulating blood; in any case, not nearly as much as it affects the reservoir blood.[1]

Due to the fact that primary haemoglobinaemia in the reservoir blood is faint, and secondary haemolysis strong, one gets the impression that different blood ingredients, previously kept apart, meet when the reservoir is emptied and thus produce secondary haemolysis.

Two years ago Knisely [1936*a*, *b*, *c*] published his investigations upon the splenic circulation. In microscope examinations of living spleens

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[1] One might suppose that the above results are no signs of a physiological process but that they have been caused by the narcotic applied. It might be conceivable that a haemolysing effect produced by the chloralose would be apparent only in the spleen depot, e.g. by a retarded destruction and therefore a prolonged effect of the chloralose in the stagnant blood of the spleen. No haemoglobinaemia, however, has been observed in control tests *in vitro* with blood containing chloralose in high concentration and which has been kept at 38–39° C. for long periods.

(mouse, rat, cat) under practically physiological conditions, Knisely noted how the erythrocytes are received and stored in the spleen sinuses while the blood fluid filtrates to the interstices of the pulp. A division of the blood into blood corpuscles and blood fluid thus takes place in the normal spleen. After passing through the pulp, the blood fluid is refiltered into the discharge veins. This author further describes how the distended sinuses, which work in shifts, discharge their content of blood corpuscles into the veins after a storage phase of from a few minutes up to 4 hr. (even up to 10 hr.; usually long times after meals). In this process the separated ingredients, blood corpuscles and blood fluid, are thus remixed and in addition intermingled with circulating passage blood.

From the theory of Bergenhem & Fåhraeus [1936] earlier cited, together with the above observations of Knisely's [1936], it may be gathered that during the reservoir phase of the spleen the separation of the erythrocytes from the blood fluid favours a lysolecithin formation in the plasma which, on the other hand, causes the decreased aggregation of the erythrocytes and the haemoglobinaemia observed in the present experiments. While it has not been directly proved that plasma—and not only highly concentrated red blood corpuscles—is retained in the spleen during the reservoir phase [cf. Barcroft, 1926*a*, and Knisely, 1936*b*, *c*], it is, nevertheless, very probable. Goreczky & Ludány [1937] have shown that the opsonin percentage in serum from the spleen reservoir blood is considerably higher than in serum from the spleen passage blood; and it would be difficult to imagine how the serum of reservoir blood could have other properties than that of passage blood if no reservoir plasma existed.

However, plasma changes of the kind found *in vitro* by Bergenhem & Fåhraeus [1936] can scarcely be the *only* cause of the strong secondary haemolysis in the reservoir blood. These authors when treating blood at body temperature for long periods found complete spherocytosis and completely annihilated rouleaux formation of the red corpuscles but never observed haemolysis.

The theory that the erythrocytes, when passing through the spleen, are normally exposed to an "Andauung", a preparation for haemolysis, which is completed in the liver [Eppinger, 1913, 1920; Naegeli, 1931], seems to have been confirmed by the present results in so far as such a preparation has been proved to take place in the reservoir blood.[1] The strong secondary haemolysis which occurred spontaneously in the reservoir blood *in vitro* seems to justify the conclusion that a spontaneous haemolysis also gradually sets in intravascularly in the reservoir blood when leaving the spleen. It is conceivable that during the rather slow passage of the blood through the liver, secondary haemolysis has time to develop to full strength and that the haemoglobin is absorbed by the liver. It is also possible that the colourless globules observed by Fåhraeus [1924] are products of the spleen haemolysis described here.

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[1] Considering the fact that the destruction of erythrocytes goes on after splenectomy, i.e. that destruction of red blood corpuscles can take place elsewhere than in the spleen, the finding of a certain stabilization in other venous blood (cf. Gawrilow [1928], and sample no. 9, Table II) and also the remarkable exception in one case with secondary haemolysis in blood from vena cava inferior (no. 9) may be of interest.

SUMMARY

The venous blood of the spleen of nineteen cats—inactive under chloralose narcosis for 3½ hr. or more—has been examined with special reference to volume percentage of red blood corpuscles, their sedimentation rate, and haemolysis.

(1) The reservoir blood of the spleen has proved to average a maximum concentration of erythrocytes of 64% (averages for vena cava inferior 44%, vena jugularis externa 41%, arteria carotis 39%).

(2) In contradistinction to the passage blood, the reservoir blood of the spleen shows a weak haemoglobinaemia and a spontaneous and comparatively strong secondary haemolysis.

(3) The haemolysis may possibly be increased during digestion.

(4) Compared with arterial or other venous blood the reservoir blood of the spleen shows a very low sedimentation reaction which is partly due to a reduced aggregation of the erythrocytes.

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REFLEX ACCELERATION OF RESPIRATION ARISING FROM EXCITATION OF THE VAGUS OR ITS TERMINATIONS IN THE LUNGS

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In a paper published in 1932 we described the reflex effects of impulses reaching the respiratory centre through the vagus resulting from changes in the volume of the lungs.

It was suggested that the acceleration of the breathing, which is the normal effect of collapse of the lungs, could not be explained by the decrease of a tonic inhibitory state, but must be due to the presence in the vagus of fibres whose terminations in the lungs are excited by collapse and which convey to the centre concerned with the rate of the respiratory rhythm impulses which augment its excitability by lowering the threshold of the chemical stimulus and so lead to an increase in the rate of discharge and consequently the rate of breathing.

In the same paper we put forward the hypothesis that the slow rhythm following vagotomy was due to the cutting off of tonic augmentor impulses normally reaching the rhythmic centre from the lungs, the removal of this influence decreasing the excitability of the centre and so causing a corresponding decrease in the rate of breathing.

We were able to show that the slowing was caused almost entirely by a great increase in the duration of the pause (second stage of expiration) and had no relation to either the depth or duration of the inspiratory phase and that it could not be ascribed to the apnoeic effect of the extreme filling of the lungs by the deep inspiratory acts.

There appeared in fact to be no satisfactory explanation of the effect of vagotomy on the frequency of the breathing or of the effect of deflation of the lungs, except that we suggested the presence of an augmentor influence arising in the lungs and conveyed to the centre by fibres, other than those carrying the inhibitory impulses. Section of these fibres causes the slow vagotomy rate, the excitation of their terminations in the lungs by collapse causes the increased rate of rhythm characteristic of a diminution in the lung volume.

In subsequent papers [1935*a*, *b*] experimental evidence was given that two functionally opposite types of nerve fibres existed in the vagus, the one carrying inhibitory, the other accelerator impulses from the lungs.

The evidence adduced was the effect of faradic excitation of the cervical vagus above and below an area of the nerve cooled to a temperature below 8° C. or subjected to compression. Excitation of the nerve above the cooled spot caused the normal inhibition of the respiration, excitation below a very marked acceleration, two effects which were identical, the first with that caused reflexly by expansion of the lungs, the second by collapse.

We deduced from this and similar experiments that the vagus contains inhibitory fibres, the passage of impulses through which is blocked readily by cold or pressure and accelerator fibres whose conductivity is only abolished at a temperature of about 1° C. and which recover much more quickly from compression. It is the section of these latter fibres which we believe to be responsible for the slow rhythm following double vagotomy.

What appears to us to be the most striking proof of the existence of these two functionally opposite types of nerve fibres arising in the lungs was the experiment illustrated in Fig. 3, p. 69 of our former paper [1935*b*], in which the vagus and superior laryngeal nerves were cooled simultaneously to the same point below 8° C., faradic stimulation of the former producing acceleration, of the latter no change in the breathing; while, when no partial blocking of the conduction by cooling was present, inhibition was caused equally by excitation of either nerve.

Other authors have, however, brought forward evidence which they believe indicates that the effects of vagotomy and the reflex acceleration accompanying collapse of the lungs can be explained without assuming the presence of more than one set of nerve terminations in the lungs the impulses from which are carried by the inhibitory fibres of the vagus.

Adrian [1933] finds that by cutting short inspiration by sudden slight inflations at some point in the inspiratory phase an increase in rate corresponding to the decrease in depth occurs. He regards this observation as supporting Head's view [1889] of the cause of the slowing and increased depth of breathing following vagotomy. The view being that after section of the vagi, the uncontrolled discharge from the centre, with the resulting greater depth and duration of inspiration, would lead to such a diminution in the excitability of the centre through exhaustion that a correspondingly longer period would be necessary for the accumulation of energy leading to the next inspiratory discharge. Thus the duration of the expiratory pause should be proportional to the depth of inspiration.

The increase in the rate of the rhythm accompanying deflation of the lungs is explained as being the result of the diminution in the state of inhibitory tone, which Adrian shows to exist in many cases (in cats) and which is still present at the ordinary level of expiration. As the lungs decrease in volume the inhibitory influence progressively diminishes and the rate of the rhythm increases proportionately.

Adrian's view with regard to the slowing following section of the vagi has been supported by Hillenbrand and Boyd [1936]. These authors in place of inhibiting inspiration at any given point by Adrian's method do so by applying a faradic stimulus to the central end of one vagus. They find that by stimulation of the inhibitory fibres of the vagus, as also of the superior laryngeal nerve, during successive inspirations a well-marked acceleration of the rhythm of respiration, accompanied by a corresponding decrease in depth, can be produced. They relate the acceleration to the decrease in the inspiratory discharge from the centre. Gesell, Steffensen and Brookhart [1937] have investigated in a similar manner the effect of intermittent stimuli applied during the progress of inspiration in vagotomized and normal animals.

They lay special stress on the fact that such stimuli increase the velocity of the discharge from the centre and greatly decrease its duration, although the resulting depth of inspiration may not be greatly altered. They consider that the more rapid completion and shorter duration of the inspiratory discharge leads to a lesser using up of energy and a corresponding increase in the rate of the rhythm, due in part to the shortening of the inspiratory phase, and in part to a corresponding shortening of the expiratory phase.

Their view seems to be that the longer the time taken to complete the inspiratory discharge from the centre, although the depth of the resulting inspiration may not be increased, the greater will be the exhaustion of the centre and the longer the expiratory pause during which recovery is taking place.

COMMENTARY AND EXPERIMENTAL RESULTS

(1) *The deflation reflex*

The increase in rate occurring is, as stated above, explained by Adrian as resulting from a decrease in a tonic inhibitory state.

The explanation seems to be definitely negatived by the fact which we have illustrated elsewhere [1935*a*, Figs. 5, 6, pp. 306–7] that after selective blocking of the conduction in the inhibitory fibres of the vagus by cold, while the normal inhibitory reflex to inflation is entirely abolished, and is in fact reversed, the acceleration due to deflation is still well marked. In regard to the view, expressed by Hillenbrand & Boyd [1936], that the reflex effect of deflation can be explained by the proportionate decrease in the depth of inspiration, or its shorter duration, we have given in a paper referred to [1932] an example (B, in Table I below) in which, with a particular degree of deflation, the rate of breathing was doubled without any change in the depth.

It seems evident that the ventilation under any given conditions, representing as it does the muscular work done in producing it, must be proportional to the energy discharge from the centre, and clearly to its condition of excitability.

In this connexion we wish here to draw attention to the importance in all observations on the respiratory function, in which complete information is desired, of recording both the rate and depth of breathing, from which data the pulmonary ventilation can be estimated and the form of the different phases of the cycle determined.

For this purpose we have used throughout a light, well-balanced bellows recorder made of thin rubberized silk or cotton material. The bellows is cylindrical in shape, the sides being encircled at intervals by thin steel wire for support. Attached to a suitable recording lever, the inertia of such an apparatus is minimal and can be relied on to give a correct record of the breathing even when, as in panting, the rate reaches between 200 and 300 per min. For dogs of from 4 to 8 kg. weight, a bellows of a capacity of 700 c.c. is suitable.

The bellows is connected to the large air reservoir into and out of which the animal breathes through valves attached to a tracheal cannula.

A stethograph on the epigastrium or the thorax gives a satisfactory record of the diaphragmatic or costal movements, but gives no information as to the combined effect of the two mechanisms on the intake and output of air.

We have frequently observed no apparent change in the depth of breathing, as indicated by the diaphragmatic movement, while significant changes in the ventilation were shown in a simultaneous volume record. With quiet breathing the costal movements are of minimal importance, in deep breathing, from any cause, their importance is greatly increased. The range of the diaphragmatic and costal movements does not in fact run parallel, and it may not infrequently be observed that the beginning of the intercostal contractions does not synchronize with that of the diaphragm.

The apparent independence of the two muscular mechanisms of breathing is of great interest, Gesell and Moyer [1935], for example, state that the intercostals and the diaphragm do not always react similarly to a reflex stimulus. They describe an observation in which on excitation of the central end of the saphenous nerve the rhythm of costal movement was double that of the diaphragm.

Coombs & Pike [1930] indeed suggest that the medullary centres governing the two functions are not identical, that for costal breathing being higher up and separate from that controlling the diaphragm.

Attention has been drawn to these facts to indicate that while a volume record obtained with suitable apparatus cannot fail to give correct information regarding the main function of respiration, a record of one or other of the two muscular mechanisms only may be misleading.

Where, as we shall show, the ventilation is much increased, so that, in the absence of any asphyxial element, a washing out of the CO_2 must occur, the chemical excitation of the centre cannot fail to be lowered. If under such circumstances an increase in the rate of the rhythm occurs, it can only imply the existence of some factor or stimulus which either raises the central excitability or directly excites the discharge.

It is this factor we have called the augmentor influence.

In Fig. 1 of the above-mentioned paper [1932] an example is illustrated of the great acceleration of the breathing produced by a certain degree of collapse of the lungs. In this case the rate increased from 18 to 93 per min., and although the depth was decreased to about half, the ventilation was increased three times (obs. C in Table I). It must be assumed that the excitability of the centre was augmented to a corresponding extent by some factor other than the normal chemical stimulus.

The figures given in the subjoined table (Table I) showing the rate of breathing, volume in c.c. per breath and ventilation per min., sufficiently illustrate the argument used in support of an excitatory origin of the respiratory response to collapse of the lungs.

TABLE I. Reflex effect of pulmonary deflation on ventilation per minute

Extra-thoracic pressure producing deflation mm. Hg.	Initial condition			During deflation		
	1	2	3	1	2	3
A, +6	15	80	1200	24	77	1848
B, +8	12	66	792	24	66	1548
C, +14	18	112	2016	93	56	5208
D, +32	30	186	5680	84	125	10500

A, B and C, dogs anaesthetized with chloralose; D, decerebrate.
Columns: 1, rate of breathing; 2, volume per breath; 3, ventilation per minute.

It will be seen that in the examples tabulated with varying degrees of pulmonary collapse, which are in cases A, B and C little more than that of normal expiration, the ventilation was greatly increased without any significant change in the depth of inspiration.

Reversal of the inflation reflex. In a paper already referred to [1935] we have discussed this phenomenon, first described by Head (*loc. cit.*), which occurs when the vagi are cooled and the lungs inflated.

The accelerated breathing seen under such circumstances in place of the normal inhibition was explained as being due to the blocking of the conductivity of the inhibitory fibres from the lungs, leaving the tonic effect on the centre of the augmentor influence alone in action.

It was therefore concluded that the terminations of the fibres carrying this influence are excited by a change in volume of the lungs in either direction.

Gesell *et al.* [1937] have described observations in which reflex acceleration of the breathing occurred with inflation when the vagi were in a normal state, the effect being exaggerated by simultaneous excitation of a sensory nerve. They believe that reflex acceleration of the breathing from changes in the volume of the lungs may occur with the vagi cut, and suggest that such reflexes may have an extra-pulmonary origin.

In fully anaesthetized or decerebrate animals we have never observed a reversal of the normal inflation reflex, except when the inhibitory fibres of the vagus have been blocked: the absence of inhibition of respiration with inflation of the lungs has, in fact, been taken as sufficient proof that the conductivity of the fibres of the vagus carrying the inhibitory impulses has been suppressed. Nor have we ever seen any reflex effect of pulmonary volume change after section of both vagi.

To test this question further, the reflex effects of inflation and collapse of the lungs have been examined in animals with artificial respiration and the thorax open; recording being by a stethograph on the epigastrium, the movements recorded being those due to the contractions of the diaphragm.

Fig. 1 is a record of such observation.

Both vagi were intact and, after the record shown in graph A had been taken, were simultaneously cooled. Graph A therefore shows the normal effects, graph B the effects after partial blocking of the conductivity of the nerves by cold.

Method. The ventilation by the respiratory pump was adjusted so that each discharge from the respiratory centre (diaphragmatic contractions) alternated with the inflation of the lungs. The discharges thus synchronizing with the pump movements. The degree of inflation of the lungs was approximately that of a normal inspiration. Inflation and deflation of the lungs were effected simply by arresting the pump at the top and bottom of the piston drive respectively. Deflation therefore corresponded to complete collapse of the lungs when the air was allowed to escape, and inflation to the change in volume such as would be produced by an ordinary inspiration and breathing arrested at the apex of the inspiratory act.

It will be seen in graph A (vagi normal) that at 1, when the lungs were in a state of slight expansion, the normal reflex inhibition of the discharge from the centre occurred, while at *D*, with the lungs collapsed, an increased rate of discharge resulted.

In graph B the pump ventilation remaining unchanged, the rate per minute of pulmonary inflations being 18, it will however be seen that when the temperature of the vagi reached 8° C. there is an immediate increase in the rate of the rhythm from 18 to 27 per min., a rate which remained constant without any apparent increase in the strength of the discharge as the temperature fell to 4°.

Fig. 1. To show inflation and deflation reflexes and reversal of the former in an animal with the open chest. Dog, 7·5 kg. Morph. 1·2 c.c. of 1 % sol. Medinal, 1·85 g. Open chest with pump ventilation. Both vagi intact. A. Vagi, normal temp. *I*, pump arrested at maximal filling of lungs (inflation); *D*, when lungs collapsed (deflation). Rate per min. of pump 18. Rate of diaphragmatic movements with deflation 32. B. Both vagi cooled to 8° and below. Change of rate at 8° from 18 to 27 per min. C. Both vagi at 5°. Pump arrested with lungs inflated at *I*, with lungs deflated at *D*. With inflation rate increased from 27 to 32 with deflation to 36 per min. 1, pump record; 2, stethograph or epigastrium; 3, carotid blood pressure (pressure in graph A, 125 mm. Hg); 4, 10 sec. Read left to right. Insp. up.

It is evident that the increase in rate is due to the removal of the inhibitory influence of the rhythmical inflations of the lungs. Had the vagi been cut at this point and all impulses arising in the lungs been prevented from reaching the centre, the slow type of respiration characteristic of vagotomy would have resulted. That the effect is not a vascular reflex is seen from the carotid blood-pressure record. The result clearly cannot be due to a decrease in the duration or extent of the discharge from the centre affecting the duration of the expiratory pause as, had this been so, some indication would be shown on the record of the diaphragmatic contractions.

The acceleration, in our view, can therefore be due only to the tonic augmentor effect carried from the lungs by vagus fibres whose conductivity is not suppressed at the temperature to which the nerves were subjected.

In graph C, the rate of discharge remaining as in B at 27 per min., the nerve being cooled to 5°, the inflations by the pump at 18. At *I*, the pump was arrested at the upper level of inflation, at *D*, in the expiratory position, the lungs remaining collapsed for about 18 sec.

At *I*, the lungs being relatively inflated, the rate of discharge at once rose to 32 per min., at *D*, with collapse, to 36.

That these changes in the rhythm are not asphyxial in origin seems evident from the fact that the change of rate is immediate and that there is no progressive increase in the strength of the discharge as indicated by the diaphragmatic movement. That they are not caused by a fall of blood pressure is seen from the record.

The fact that the thorax is open, the lungs remaining for the period of rest at constant volume (which is even in the inflated state at *I*, con-considerably below their normal volume with the closed chest), removes any likelihood of the effects being of extrapulmonary origin.

The only explanation remaining is that the acceleration observed in both conditions is of reflex origin arising from the tonic excitation of augmentor nerve terminations in the lungs.

(2) *The cause of the vagotomy effect*

We have previously drawn attention to the fact that the cycle in the vagotomized dog has the same characteristics as in the intact animal (anaesthetized or decerebrate). In both the rate of rhythm is mainly dependent on the duration of the pause, the second phase of expiration; the duration and depth of inspiration having little relation to the rate, or, consequently, to the duration of the pause. An example illustrating this is seen in Fig. 2 B and C of our paper [1932], and three examples are given in Table I of that paper showing the same fact by a comparison of the duration of the different phases and the depth and rate of breathing at different periods after vagotomy.

It is difficult to explain, on the hypothesis that the slow rate of the rhythm after vagotomy is due to the exhaustion of the centre caused by the uncontrolled discharge, the fact, shown in the figure referred to, that, while the depth of inspiration recorded 30 min. after section was reduced to only half the initial (soon after section), the duration of the inspiratory phase, the rate of breathing and the length of the expiratory pause were scarcely changed. It is evident also, if Head's hypothesis, as supported by the later observers mentioned, were valid, that (*a*) the change in the depth of inspiration should be accompanied by a corresponding increase in the duration of the recovery phase (the expiratory pause). Reference has been made above (p. 504) to evidence adduced in our former paper [1932] that this relationship does not occur; and (*b*) that blocking of the passage of inhibitory impulses from the lungs should produce the same change in the breathing as section of the two vagi. That this is not the case is shown by the observation illustrated in Fig. 2, which is a record of the results accompanying gradual cooling of both vagi from 13 to 0° C.

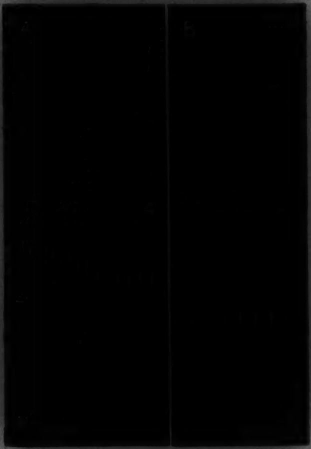

Fig. 2. Effect of gradual cooling of both vagi on the rate and depth of breathing. Dog, 6 kg. Morph. 1 c.c. of 1% sol. Medinal, 1·32 g. A. Record during cooling from 13 to 4°. B. Terminal part of record. Temperature of nerve 0 to $-2°$. Rate of rhythm: at 13°, 24; at 8°, 20; at 4°, 14·5; at 0°, 10 per min. 1, carotid blood pressure (about 120 mm. Hg); 2, bellows record; 3, 10 sec.

It will be seen in graph A that although there is some increase in depth and decrease in rate below 8°, the breathing characteristic of vagotomy is not seen until the temperature has reached 0° when all conduction in the nerve has been suppressed.

Steffensen, Brookhart & Gesell [1936] describe observations on the effect of cooling on the conductivity of the vagus fibres, a type of thermode being employed in which 4·5 cm. of the nerve is exposed to cold. They find that a progressive diminution in the conductivity occurs, beginning as soon as the temperature falls below body temperature, both the inhibitory and the augmentor fibres being affected. Their evidence of this is the effect of gradual cooling of the nerve on the slowing of breathing with inflation and the acceleration with deflation of the lungs.

Using the type of thermode we have described, in which not more than 1·5 cm. of the nerve is cooled, we have never observed any indication that the inhibitory fibres are affected at a temperature above 12° C., while below 8° C. their conductivity appears to be completely blocked, since below this point there is no evidence of the passage of inhibitory impulses arising either from the physiological stimulus of pulmonary inflation or from faradic excitation of the central end of the nerve.

As regards the augmentor fibres there is some evidence of a gradual decrease in conductivity until they are completely blocked at a little above 0° C. This no doubt accounts for the relative slowing of the rhythm seen in the figure between 8 and 4° C. (graph A, Fig. 2).

(3) *The effect of exciting the vagus in which the conduction of the inhibitory fibres has been blocked*

Hillenbrand & Boyd express the view that the acceleration of the breathing we have described under such conditions is related to a coincident decrease in the extent of the inspiratory discharge.

Their view appears to be negatived by the figures given in Table II of the rate and depth of breathing and ventilation per minute resulting from stimulation of the vagus.

TABLE II. Rate and depth of breathing and ventilation per minute with excitation of the central end of vagus with inhibitory fibres blocked by pressure or cooling

	Initial state of breathing			During excitation		
Procedure	1	2	3	1	2	3
A. Left vagus intact. Right vagus compressed and excited	18	75	1350	42	81	3400
B. Vagotomy. Right vagus compressed	12	300	3600	22	240	5280
C. Left vagus intact. Right vagus cooled	24	144	3450	48	120	5760
D. Vagotomy. Right vagus cooled	4	150	1000	66	44	2900
E. Open chest. Left vagus intact. Right vagus cooled and a pulmonary branch excited	24	—	—	56	—	—

Observations A and B on the same animal. C, D and E, different animals. Anaesthetic in all cases medinal 0·22 g. per kg.

Columns: 1, rate of breathing; 2, inspiratory volume in c.c.; 3, ventilation per minute.

It will be seen from these figures that in some cases there is no significant decrease in the depth of inspiration when the vagus is excited below a spot at which the passage of inhibitory impulses has been blocked, either by compression or cooling, and that judged by the ventilation, which is always much increased, there is no proportion between the inspiratory discharge and the duration of the cycle which could in any manner account for the increased rate of the respiratory rhythm. It is evident that the greatly increased inspiratory discharge is an excitatory phenomenon, and is best explained as being due to the stimulation of the augmentor fibres which we believe to be present in the fibres of the cervical vagus originating in the lungs.

The argument in favour of the acceleration being due to the excitation of specific nerve fibres is, in fact, the same as that previously used in regard to the deflation reflex, based on the figures for ventilation given in Table I of this paper.

(4) *The respiratory response to excitation of a sensory nerve*

Gesell *et al.* [1937] suggest that the acceleration of the breathing we describe as resulting from stimulation of the central end of the vagus below a spot partially blocked by cooling or pressure is due, not to the excitation of specific augmentor fibres arising in the lungs, but to the excitation of fibres in the vagus carrying sensations allied to pain, and corresponds to the increase in the rate of the rhythm accompanying stimulation of any sensory nerve such as the saphenous.

In observations [1935*b*] bearing on this point we showed that while excitation of the thoracic vagus below the hilum of the lungs, that is below the entry of the cardiac and pulmonary branches, containing only fibres from the abdominal viscera, the cervical vagus being cooled to below 8° C., produces no change in the breathing, yet a pulmonary branch isolated from its path on one of the bronchi caused, under similar conditions, the same marked acceleration as we were accustomed to find with the partially blocked cervical vagus. In the absence of partial blocking excitation of this pulmonary branch caused the usual inhibition.

We have examined the effect of excitation of the sciatic and saphenous nerves by continuous faradization and by faradization confined to the inspiratory phase. A short account of our observations was given at the meeting of the Physiological Society in July 1938. We find in both cases the classical effects, namely, a great increase in the rate and depth of breathing accompanied by a marked rise of blood pressure. With intermittent stimulation, the same increase is seen as with the vagus [Gesell *et al.* 1937] but without the decrease in depth.

The above is true of the anaesthetized animal. If, however, the excitation is carried out on the same nerves in the decerebrate preparation, the section being made slightly posterior to the junction of the mid-brain and cerebral hemispheres, no significant change in the rate of the rhythm occurs. On the other hand, excitation of the partially blocked vagus causes the same marked acceleration as in the anaesthetized animal.

Fig. 3 illustrates the effect of faradizing the sciatic in the anaesthetized animal A, and the decerebrate B and C.

Faradic excitation of the central end of the sciatic (or other sensory nerve), whether continuous or confined to either phase of the cycle, has always, in our hands, produced the characteristic response, namely, a great increase in the rate and usually the depth of breathing in the intact animal.

Fig. 3. Respiratory response to excitation of the sciatic nerve before and after decerebration. A and B. Dog. 11. i. 38. Morph. Medinal anaesthesia. Between A and B decerebration was carried out through the frontal 1/3 of the ant. colliculi. A. Faradic excitation of the central end of sciatic. Coil at 12 cm. B. The same after decerebration. C and D. Dog. 12. iv. 38. Decerebrate. Double vagotomy. C. Excitation of sciatic during inspiratory phase. Coil at 7 cm. D. Excitation of cervical vagus during inspiratory phase. 1, carotid blood pressure; 2, bellows volume record; 3, stimulation signal; 4, 10 sec. Read left to right.

The above records show that, in the decerebrate animal, the respiratory response to excitation of the sciatic nerve is abolished (graphs B and C) but is unaffected in the case of the vagus (graph D).

This is what would be expected if, as is generally stated, the centre for the reception of pain stimuli lies anterior to the point selected for decerebration; while the centre originating the acceleration with vagus stimulation must lie in the mid-brain or medulla and may be assumed to be that part of the respiratory centre from which the rhythm arises. It seems evident therefore that the fibres in the vagus, the stimulation of which causes an increase in the rate of the rhythm, are functionally different from those causing a similar effect carried in the somatic sensory nerves.

To verify this distinction we have examined the effect of cooling on the conductivity of sensory nerves in regard to the respiratory response to faradization, the nerves examined having been the sciatic, the peroneal and the saphenous nerves. In each case it was found that if the nerve was excited below a point cooled to between 6 and 9° C. the rapid breathing normally occurring was absent, while excitation immediately above the cooled area gave the usual result.

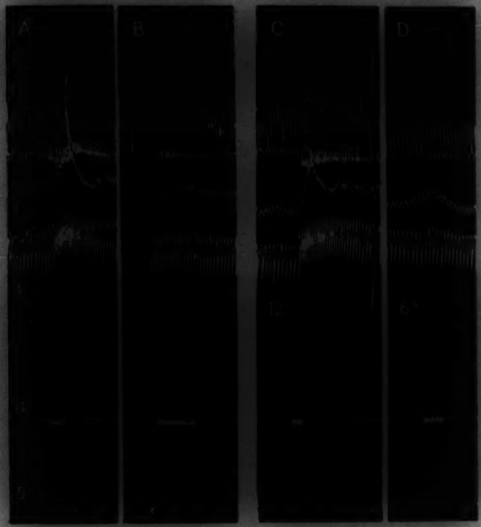

Fig. 4. Showing the effect of cooling below 8° on the respiratory response to excitation of the sciatic nerve. Dog. 6·5 kg. Morph. 0·3 c.c. 1 % sol. Medinal 1·32 g. A and B. Sciatic cooled to 7°. Two electrodes, one above, the other below the cooled spot. A. Excitation above the cooled spot. B. Below. C and D. Sciatic gradually cooled from 12 to 6°. Electrode in both below the thermode. C. Temperature of cooled area 12°. D. 6°. 1, stethograph, insp. up; 2, carotid blood pressure; 3, bellows volume record, insp. down; 4, stimulation signal; 5, 10 sec. Read left to right.

Fig. 4 illustrates this observation. Graphs A and B show the effect of a faradic stimulus to the central end of the sciatic nerve A above and B below an area cooled to 7° C. In the observation shown in graphs C and D the electrodes were in both cases below the thermode; in C, however, the temperature was 12°, in D 6° C.

In Graphs B and D not only is the respiratory response absent but also the great rise of blood pressure seen in graphs A and C is very much diminished. It appears that the conductivity of the afferent fibres carrying the impulses responsible for both types of response is, like the inhibitory fibres of the vagus, blocked at temperatures below 8° C.

It is of interest to compare the results shown in the figure with Grützner's observations [1878]. This author found that the conductivity of the motor fibres of the sciatic nerve in the dog was blocked at a temperature of 8° C. and that the afferent fibres carrying the impulses causing the reflex rise in blood pressure were blocked, with stimuli of moderate strength, at 10°. He does not refer to the respiratory response to excitation of this nerve but regards the vascular reflex as evidence of conduction along sensory fibres.

There is in fact no significant difference between our results and those obtained fifty years earlier by Grützner.

It may be noted in this connexion that Howell, Budgett & Leonard [1894], who examined the effect of cold on the conductivity of the vagus, state that the passage of impulses causing sensory reactions is blocked if the nerve be cooled to 10° C. The anaesthetic used by these authors was morphine and chloral hydrate, the anaesthesia being probably not very deep.

(5) *Excitation of the central end of the vagus during one phase of the cycle*

Hillenbrand & Boyd [1936], in discussing our observations on the respiratory accelerator fibres of the vagus [1935*a*, *b*], raise the objection that continuous and not intermittent faradization was employed. We cannot follow the meaning of this criticism. Indeed, the same objection might be raised to a statement that continuous faradic excitation of the normal unblocked vagus causes inhibition of the breathing. In any case it will be shown later that acceleration of the breathing can equally well be obtained, the vagus being cooled, by excitation during the expiratory phase, as by continuous excitation.

The effect of intermittent stimulation of the vagus during the inspiratory phase as observed by the above authors, and by Gesell *et al.* [1937] is of considerable interest and in some respects not easy to explain.

Following the publication of Adrian's paper [1933] we made some observations (unpublished) on the effects of faradizing the central end of the vagus during inspiration.

We found in some cases that by cutting short this phase of the cycle so as to decrease considerably the depth, there was a definite increase in the rate of the rhythm. This seemed to us to be explained by the effect of the decreased ventilation and possibly, following Adrian, by the lessened consumption of energy involved in the diminished inspiratory discharge.

An acceleration due to either of these causes might very well result from the stimulation of inhibitory fibres either in the vagus or the superior laryngeal nerves.

It will, however, be shown below that neither of these explanations is applicable, the first as the effect is immediate, the second because there is no relation between the change in the rate of the respiratory rhythm and the depth or duration of the inspiration.

Experimental observations. The animals used were dogs, in the majority of cases anaesthetized with medinal, in some cases decerebrate.

Recording was by the bellows recorder and stethograph; the carotid blood pressure was in most cases registered.

Method of automatic intermittent excitation. Connected by a side tube to the tracheal cannula was a small light bellows actuating a lever pivoting in the middle of its length, at each end a wire being attached dipping as the lever descended at either end into a mercury cup, the contact so made closing the primary circuit of the inductorium.

The circuit would thus be closed during inspiration by the contact at one end of the lever, during expiration at the other.

By means of a two-way switch the current could be made to pass through the inspiratory or expiratory contact at will, excitation of the nerve therefore taking place either during the inspiratory or expiratory phase as desired.

RESULTS

Stimulation of the central end of one vagus, whether the other nerve is intact or has been cut, during the respiratory phase, always leads to an increase in the rate of the rhythm which is frequently considerable (e.g. three times the initial) with even weak currents. (As in graph A in Fig. 6, p. 516 below.)

There is a variable decrease in the depth of inspiration, but even with strong stimuli it has, in our hands, seldom occurred that the depth was decreased by more than 50 %. There is, in fact, no relation between the extent of the inspiratory discharge and the rate of breathing set up. In consequence the ventilation is in all cases increased, being sometimes more than doubled (see Table III). On the other hand, faradization during the brief first phase of expiration, and more markedly during the pause, always causes a considerable slowing of the rhythm (Fig. 6 B). Examples of the effect of exciting the central end of the normal vagus during the inspiratory phase of the cycle, and, in D′ the expiratory, on the frequency of breathing and the ventilation per minute, are shown in Table III.

TABLE III. Effect on the breathing of faradic excitation of the vagus, confined to one phase of the cycle

	Initial condition		During excitation	
	Rate	Ventilation	Rate	Ventilation
A. Stimulus with inspiration	12	4200	24	6600
B. ,, ,, ,,	28	2240	60	3300
C. ,, ,, ,,	24	1800	48	2400
D. ,, ,, ,,	12	2100	30	4200
D′ Stimulus with expiration	12	1800	8	1440

A. Dog. Decerebrate with medinal anaesthesia (graph D, Fig 3, p. 508).
B. Dog. Medinal anaesthesia (graph C, Fig. 6, p. 516).
C. Dog. Medinal. Open chest. Pulmonary branch of vagus stimulated (not figured).
D. Decerebrate dog (graph A, Fig. 6, p. 516).
D′. Same animal (graph B, Fig. 6, p. 516).

Superior laryngeal nerve

Hillenbrand & Boyd [1936] describe a similar acceleration of the respiratory cycle on faradizing the central end of the nerve during the inspiratory phase of the cycle and regard it as due to the cutting short of the inspiratory phase by the excitation of inhibitory fibres and the after effect of this on the centre and the duration of the expiratory pause.

Continuous stimulation of the superior laryngeal nerve in the fully anaesthetized or decerebrate animal, in our experience, always arrests the breathing in the expiratory position, only reactions inhibitory of respiration occurring without active expiratory or other phenomena.

We have repeated their observations but have failed to obtain any significant increase in the rate of the rhythm, more frequently a slowing, on excitation of the central end of the nerve during inspiration, although cutting short of that phase is constantly seen.

Fig. 5 shows a record of the effect of faradizing the superior laryngeal nerve during the whole cycle, and during the inspiratory phase only, and the comparative effect of the latter procedure when carried out on the vagus.

It will be seen that continuous excitation of the nerve produces what we consider the normal inhibitory effect (graph A), excitation during the inspiratory phase causing a marked cutting short of inspiration accompanied by a definite slowing of the rhythm, the ventilation being about halved. On the other hand, during similar stimulation of the vagus, while there is some diminution in the depth of breathing, the rate of the rhythm is more than doubled, and the ventilation increased by a little more than 30%.

Continuous excitation of the vagus would produce an inhibitory effect identical with that shown in graph A with this type of excitation of the superior laryngeal.

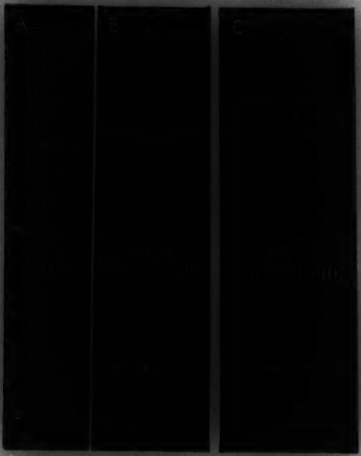

Fig. 5. Showing the effect of exciting the superior laryngeal nerve during the inspiratory phase of breathing and of the vagus for comparison. Dog. 7 kg. Morph. 0·7 c.c. of 1% sol. Medinal 1·55 g. Left vagus intact. Right superior laryngeal and vagus cut and excited. A. Continuous faradic excitation of superior laryngeal nerve. B. Excitation during inspiratory phase of superior laryngeal nerve. C. Excitation during inspiratory phase of vagus. In B, initial rate of rhythm per min. 33; ventilation 2475 c.c.; during excitation 25; ventilation 1250. C. Initial rate of rhythm 28; ventilation 2240; during excitation 60; ventilation 3300. 1, carotid blood pressure (in graph A, 120 mm. Hg); 2, bellows volume record; 3, stimulation signal; 4, 10 sec. Read from left to right. Insp. down.

During the period of excitation in graph B there is no change in the duration of the inspiratory phase of the cycle, the decrease in rate being due to a prolongation of the second phase of expiration.

There can be no doubt that the effects, shown in the figure, of stimulation of the superior laryngeal, are due to the excitation of inhibitory fibres, it can only be concluded that the acceleration seen under similar conditions with the vagus has an excitatory origin.

The observation seems to negative the view put forward by other observers, referred to in this paper, that the cutting short of the inspiratory discharge from the centre by the excitation of inhibitory fibres can produce a corresponding shortening of the expiratory phase and an increase in the rate of the rhythm, or, conversely, that an increase in the extent of inspiration, as in vagotomy, can in the same way account for the characteristic slowing.

The superior laryngeal nerve undoubtedly contains fibres conveying painful and other sensory impulses, and we have frequently observed sensory reactions on exciting this nerve, including rapid respirations, an increase of inspiratory tonus or active expiratory movements, results similar to those described by the above-mentioned authors.

These effects only occur, however, if anaesthesia is light, and are absent in fully anaesthetized animals. We are therefore of the opinion that the increased rate of the rhythm and other respiratory responses to excitation of the superior laryngeal nerve, described by Hillenbrand & Boyd, must be ascribed to the lightness of the anaesthesia employed.

The figures, tabulated below, from an observation in which the superior laryngeal and the vagus were excited first, when the animal was anaesthetized just sufficiently to abolish evidence of consciousness to pain, and secondly, when fully anaesthetized, appear to support this conclusion.

TABLE IV. Comparative effect on respiration of exciting the superior laryngeal and vagus nerves, respectively, in a dog under light and full anaesthesia

	Initial condition			Ditto during excitation		
Superior laryngeal	1	2	3	1	2	3
Light anaesthesia	7	650	4550	30	75	2250
Full anaesthesia	10	500	5000	7	450	3150
Vagus						
Light anaesthesia	7	580	4060	60	125	7500
Full anaesthesia	9	500	4500	24	250	6000

Columns: 1, rate; 2, depth of breathing; 3, ventilation per minute.

It will be seen that in the case of the superior laryngeal with light anaesthesia, the rate of breathing was increased four times, while with full anaesthesia the rate decreased from 10 to 7 per min., the ventilation being considerably diminished in both conditions, and the comparative figures for the vagus showing a greatly increased rate of rhythm and a considerable increase in ventilation, with both light and deep anaesthesia.

Intermittent excitation of the partially blocked vagus

The effects of such excitation of the vagus during either phase of the cycle have been described above (p. 506). An example of the reflex acceleration of the breathing produced by stimulation during inspiration is shown in Fig. 3D above (p. 508), and details are given from that and other observations in Table III (p. 512).

In view of the great increase in the rate of the rhythm with faradization during the inspiratory phase and the much increased ventilation (see Table III) it is difficult to avoid the conclusion that an excitatory factor is involved and not an inhibitory. It seemed therefore of interest to carry out the same procedure on a nerve in which the conduction in the inhibitory fibres had been blocked by cooling below 8° C.

Fig. 6 illustrates such an observation in which the effects of intermittent excitation of the normal vagus and the partially blocked nerve are compared.

In the figure, graphs A and B show records of the results of exciting the normal vagus; A during inspiration, B during the expiratory phase. In the first a marked acceleration is seen, in the second considerable slowing.

Graphs C and D record the reflex effects of exciting the cooled vagus in a similar manner. The result is strikingly different. It will be seen that excitation during the inspiratory phase of the cycle leads to no change in the rate of the rhythm, but some increase in the depth of the breathing, excitation during the expiratory phase being accompanied by a very marked acceleration of the rate.

The slowing of the breathing with excitation during expiration of the normal nerve is evidently due to the stimulation of the inhibitory fibres, and it is evident also that the acceleration seen on excitation of the cooled nerve during the same phase arises from the stimulation of the augmentor fibres, the conductivity of the inhibitory fibres having been abolished.

The unexpected result of excitation of the partially blocked vagus during inspiration, namely, the absence of the accelerator effect which occurs when the normal nerve is similarly excited, or with continuous faradization of the cooled nerve, makes it difficult to find an equally simple explanation of the increased rate of rhythm accompanying stimulation of the normal nerve during the inspiratory phase.

That the phenomenon is not due to excitation of augmentor fibres is evident, for, as has been just stated, it is absent under conditions when only the inhibitory fibres are blocked. Nor in view of what has been said above (p. 512) in discussing the effect of intermittent stimulation of the superior laryngeal nerve can it originate from the excitation of inhibitory fibres (as suggested by Hillenbrand & Boyd).

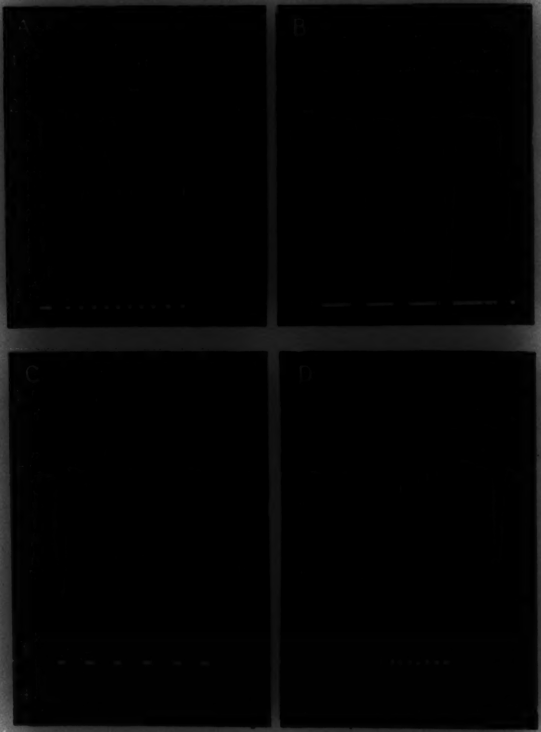

Fig. 6. Showing the effects of excitation of the normal and partially blocked vagus during the inspiratory and expiratory phases respectively. Decerebrate dog. A and B. Normal vagus. A. Faradic excitation during inspiratory phase. B. During expiratory. C and D. Nerve cooled to 5°. C. Excitation during inspiratory phase. D. During expiratory. Ventilation in A: initial 2100 c.c. per min.; during excitation 4200 c.c. Ventilation in D: initial 2100 c.c. per min.; during excitation 4290 c.c. 1, carotid blood pressure; 2, bellows record; 3, stimulation signal; 4, 10 sec. Read left to right. Insp. down.

That the acceleration is of excitatory origin cannot be doubted in view of the greatly increased ventilation (see Table III).

The question however arises as to the nature of the fibres of the vagus which are neither inhibitory nor augmentor, but whose conductivity is, like the inhibitory and the sensory fibres of a somatic nerve (p. 509 of this paper), blocked at a temperature of about 8° C.

The accelerator reflex to excitation of the vagus during inspiration occurs equally well in the decerebrate as in the anaesthetized animal, thus differing from the respiratory response to painful stimuli and implying that the fibres carrying the impulse terminate in the mid-brain or medulla. That they are not of extrapulmonary origin is shown by the fact that similar excitation of a pulmonary branch of the vagus gives the same accelerator response.

DISCUSSION

The question at issue in this paper, namely, whether the effects described can be explained as originating from the excitation of a single type of nerve ending in the lungs or the corresponding fibres of the vagus, or whether the presence is required, as we believe, of a second augmentor type, has been discussed under different headings, and the conclusion reached that the phenomena cannot be explained except on the latter assumption.

Section 1 deals with the deflation reflex and the reversal of the inflation reflex under certain conditions.

Section 2 deals with the view that vagotomy slowing is due to the cutting off of an augmentor influence reaching the centre from the lungs.

Section 3 shows the effect of exciting the partially blocked vagus.

Section 4 gives a comparison of the respiratory accelerator response to excitation of a sensory nerve and of the vagus.

Section 5 shows the reflex effect of stimulating the vagus during one phase of the cycle.

With each section evidence is given that the effects described are excitatory in nature and depend for their production on an augmentor influence which raises the excitability of the respiratory centre.

The alternative explanations of other observers have been considered in relation to this evidence.

Adrian's view [1933] that the accelerated breathing accompanying collapse of the lungs is due to the decrease of a tonic inhibitory state was based on the apparent absence of electrical changes in the vagus at the level of expiration or during deflation. This was discussed in our previous paper [1935*b*, p. 71], where attention was drawn to the observations of

Hammouda & Stella [1935] who were able to demonstrate discharges of measurable activity under such conditions.

The crucial evidence against Adrian's view is given in § 1 of the present paper, namely, that after the inhibitory inflation reflex has been abolished by selective cooling of the vagus, the deflation reflex is still active. That the accelerated breathing under discussion is not a reflex of vascular origin was discussed in a previous paper [1935*a*, p. 293]. Moreover, the blood-pressure records in various figures in this and our other papers indicate that where a change of pressure occurs it is in the direction of a rise, the normal response to which would be a decrease and not an increase in the rate of the rhythm.

The most important alternative explanation of the augmentor effects and the slowing following vagotomy, put forward by Adrian, Gesell and his co-workers, and by Hillenbrand & Boyd, is based on Head's [1889] original hypothesis of the influence of the energy discharge from the centre during inspiration on the subsequent rate of recovery during the expiratory phase.

As Gesell expresses it [1938], "Depth and rate of breathing are two separate phenomena yet inextricably connected." This is of course true, as it is on the co-ordination of these factors that the respiratory function of the ventilation of the lungs depends; but the connexion is due to the common reflex or direct action of changes in the composition of the blood on the respiratory centres, not on any influence the inspiratory discharge may have on the duration of the expiratory pause.

What seems to be convincing evidence that the depth and velocity of inspiration in no way governs the rate of the rhythm is given in the figures and tables attached to the different sections of this paper. That this applies equally to the slowing following vagotomy was shown on pp. 86 and 90 of our original paper [1932] and also to the slowing of normal breathing produced by slight inflation on p. 102 of the same paper.

The main argument used in the present paper in support of the view that the phenomena described are of excitatory origin is based on an analysis of the records shown in the various figures, and an estimate of the changes in ventilation found.

Were it true that a reflex increase in the rate of the rhythm could be explained by a coincident decrease in the depth or duration of the inspiratory phase, the ventilation should, *a priori*, remain unchanged. When, however, the increase in rate is accompanied by a significant increase in the ventilation, this must imply a stimulus which raises the central excitability. The increased ventilation seen in asphyxial conditions is due, in part, to an increase in the central stimulus, in part to a reflex increase in excitability of the centre caused by impulses reaching it from the peripheral chemo-sensitive areas. Similarly, in our view, the central excitability may be raised without any change in the composition of the blood by reflex augmentor impulses originating in the lungs, the ventilation being thereby increased, just as the excitability of the centre may be decreased and the ventilation diminished by inhibitory influences.

The only distinction we find [Hammouda & Wilson, 1932, p. 112] is that the augmentor influence appears to act only on the part of the centre responsible for the rate of the rhythm. We have never seen any evidence that reflex stimuli reaching the centre through the vagus cause an increase in the depth of breathing, with the possible exception of the excitation of the vagus during the inspiratory phase.

With the different sections tables are given showing the rate and depth of breathing and the pulmonary ventilation per minute observed in the various procedures. The greatly increased ventilation coincident with the deflation of the lungs or with the excitation of the vagus when the passage of inhibitory impulses has been blocked supply, on this view, striking evidence of the central excitatory nature of these phenomena.

In § 3 the suggestion that the reflex acceleration of the breathing produced by excitation of the cooled vagus may not be due to stimulation of augmentor but of sensory fibres, and is comparable to the respiratory response to pain, has been fully considered, evidence being given that appears to negative the suggestion.

We have discussed on pp. 293–5 of a previous paper [1936] the origin of the conflicting results of vagus excitation described by the early observers, results which the effects obtained by the authors quoted in § 5 p. 514 in many cases closely resemble. It was suggested there that the rapid breathing and inspiratory spasm stated to occur might be accounted for by the ineffective anaesthetics, such as opium and chloral, used by observers at that time; and that the reactions described originated in regions above the mid-brain, probably in the hypothalamus.

We have drawn attention to the possibly misleading results of observations on the conscious human subject in two previous publications [1933, p. 262, footnote; 1936, p. 291].

It is of course beyond question that, under ordinary conditions of life, the breathing is controlled and adapted to postural or other changes by impulses originating in the forebrain. The absence of the normal response to deflation and inflation with postural change is an example. One of the present writers [W. H. W. 1927] showed that on assuming the recumbent posture there is a marked decrease in the volume of the lungs (reserve air measurement), amounting in some individuals to as much as 1000 c.c. Accompanying this great decrease in the lung volume there is no indication of an increase in the rate of breathing, nor on again rising to the erect position is there any sign of an inhibitory effect.

In conscious or lightly anaesthetized animals such influences conflict with the pure vagus effects; they arise or are relayed to the respiratory centre from centres almost certainly in the hypothalamus.

The importance of this area in respiration may be seen from the observations of Crouch & Elliot [1936], who find, in addition to a centre governing the heat-regulating mechanism of panting, two regions, one anteriorly, the other farther back, either of which, when excited, produce rapid and deep breathing.

Similarly, Beattie [1938] states that an area lying around the anterior commissure gives rise when stimulated to a decrease in the depth and rate of breathing, and a region in the posterior part of the hypothalamus which has the opposite effect.

It is clear that in the study of the function of the vagus in respiration, the complicating effect of such sensory or other reflexes, however great may be their occasional importance, must be eliminated.

In the decerebrate animal in which the brain stem has been divided between the anterior colliculi and the thalamic area, the breathing shows no abnormality, the respiratory centres reacting normally to changes in the composition of the blood, and consistently to the natural stimuli arising in the lungs or to faradic excitation of the vagus. The same may be said of animals under full surgical anaesthesia, the corneal reflex being still active but all sign of sensory reactions absent, except that with decerebration the possible influence of the anaesthetic is also eliminated.

The cause of the characteristic change in the breathing, resulting from vagotomy, has been considered in § 2: there are, however, some points of interest which have not been discussed.

Following section of both vagi there is an immediate great increase in the depth of inspiration and prolongation of the expiratory phase, the ventilation being commonly more than doubled. After a varying interval the breathing stabilizes itself with a very marked diminution in depth, the rate remaining either unchanged or slightly slower, and the ventilation generally below the pre-vagotomy volume (see tables, pp. 86 and 90, 1932).

That the final result is not a transitory phenomenon is shown by Pavlov's observation [1896] that dogs can be maintained in good health for more than 12 months after section of both vagi, the slow rate of the rhythm established immediately after section showing no significant change.

That the respiratory centre is comparatively insensitive to the chemical stimulus after vagotomy is generally admitted. This, however, applies more to the part of the centre governing the rate of the rhythm than to the lower centre upon the activity of which the depth of breathing depends; in asphyxial conditions the depth rapidly becomes maximal, the change in rate being less marked. (An example is shown in graph D, fig. 2, p. 86 [1932].[1]) This we have ascribed to the absence of the tonic central excitatory effect of the augmentor influence from the lungs. In the present paper we have adopted the view that whether caused by chemical or nervous influences, increased ventilation implies an augmentation of the central excitatory state.

It is of interest, therefore, to consider whether the exaggerated depth and consequent hyperventilation in the initial stage of post-vagotomy breathing is sufficiently explained by the release of the centre from the normal vagal inhibitory reflex action, and how the production of the second stage, in which the depth of inspiration is much diminished, and the ventilation usually reduced to a subnormal level, can be accounted for. In the example referred to above, the normal ventilation was 1260 per min., during the first post-vagotomy stage 2000 c.c., becoming stable after 30 min. at 900 c.c.; the long period elapsing before the establishment of the second stage excludes acepnia as a cause.

Kramer has shown by the photoelectric method [1936] that even during normal respiration the oxygen saturation of the arterial blood shows a measurable decrease at a point corresponding to the end of expiration, while during the prolonged expiratory phase, as after division of the vagi, the oxygen content of the blood sinks to a point much below that of normal arterial blood, the CO_2 content probably varying equally in the opposite direction. This is not surprising considering that, as in the example given, venous blood was passing through the lungs during ten heart beats without any renewal of the air in the alveoli.

It is evident that during the slow rhythm the centre, and other areas responsible for the chemical stimulus to respiration, are receiving blood at some period of the cycle which differs little in its gas content from venous blood.

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[1] In that observation with rebreathing while the ventilation increased from 900 to 4400 c.c. per min., the rate increased only from 4·5 to 8 per min.

Heymans & Bouckaert [1938] have recently found reasons for believing that the nerve endings in the glomus caroticus have a lower threshold of excitability for changes in the CO_2 content of the blood than the respiratory centre, that their excitation and the reflex response is immediate but that adaptation soon takes place. They find, on the other hand, that while the effect on the respiratory centre is slow in developing, it is prolonged.

In the light of these observations of Kramer and Heymans it is suggested that an excitatory element, namely, the low periodic level of aeration of the blood acting on the peripheral chemo-sensitive areas, is in part responsible for the hyperventilation seen as the initial effect of division of the vagi. The lowered ventilation of the second stage would follow from the gradual adaptation of the nerve endings of the glomus, and the fact that the response of the respiratory centre itself is depressed by the absence of the augmentor influence from the lungs.

Whether or not the above deductions from the work of these authors are justified, it cannot be physiologically beneficial that the tissues, and especially the heart muscle, should be supplied for a period of possibly five heart beats, as may happen after vagotomy, with blood very markedly deficient in oxygen, even though the total oxygen intake per minute may be normal.

These wide fluctuations in the composition of the blood are absent with the rhythm of normal breathing, and their absence is due to the influence of the vagus impulses reaching the centre from the lungs.

We venture to suggest that this is the primary function of the pulmonary vagus.

The question is clearly one which must be the subject of further investigation.

In conclusion, the problem remains of the origin of the accelerated rhythm accompanying faradic excitation of the vagus during the inspiratory phase. In § 5 it has been shown that it is not related to a cutting short of that phase and is excitatory in nature. It is also shown that the effect is not due to the stimulation of either inhibitory or augmentor fibres.

The nerve fibres which when excited give rise to the accelerated breathing are shown to be of intra-pulmonary origin and to have the same reaction to cold as the inhibitory fibres of the vagus and the afferent fibres of somatic nerves. That they are not concerned with the conveyance of impulses akin to those of pain is shown by the fact that the reflex occurs as well in the decerebrate as in the anaesthetized animal.

It might very well be concluded that the effect is due to the excitation of afferent fibres in the vagus of a third functional type. This may, however, not be a necessary conclusion, as the possibility is not excluded that while stimulation of one or other of the two functional types we have described does not give rise to the phenomenon their simultaneous excitation may do so.

The solution of this problem is thus left for further investigation.

SUMMARY

1. The thesis is adopted that an increase in the pulmonary ventilation, whether this be due to direct or indirect effect of the chemical stimulus, or to the excitation of nerve endings or nerve fibres, implies an increase in the energy discharge from the centre.

2. No evidence is found that the rate of discharge from the centre is governed by the energy developed in each inspiratory discharge, or, in other words, that the duration of the expiratory phase of the respiratory cycle is a function of depth or duration of inspiration. Hence it is concluded that neither can the reflex acceleration of the breathing be explained as resulting from the cutting short of the inspiratory discharge caused by the excitation of inhibitory nerve endings or fibres, nor can the slowing after division of the vagi be explained on the basis of Head's original hypothesis.

3. It is shown that the reflex increase in the rate of the respiratory rhythm accompanying deflation of the lungs or excitation of the central end of the vagus (selectively cooled) is not due to stimulation, in either case, of sensory fibres such as are found in somatic sensory nerves. Evidence is also referred to showing that neither of these reflex effects is related to a fall of arterial blood pressure or in the former to the decrease of an inhibitory tonic influence arising in the lungs.

4. The respiratory response to excitation of the central end of a somatic sensory nerve is described. It is shown that such responses arise in regions of the nervous system above the mid-brain. In this connexion attention is drawn to the need in carrying out investigations of the vagal reflexes from the lungs of employing fully anaesthetized animals and of confirmatory observations on the decerebrate preparation to avoid the complicating and misleading effects of such sensory reactions.

5. The effects of excitation of the central end of the vagus restricted to one phase of the respiratory cycle are described; and the cause of the accelerated rhythm accompanying stimulation during the inspiratory phase discussed.

6. The observations recorded in the present paper, with the examination of the views expressed by other authors, confirm our previous conclusion that two functionally different types of vagus nerve endings exist in the lungs, the one inhibitory of respiration, the other augmentor of the rate of the rhythm.

7. It is suggested that, while changes in the composition of the blood, acting reflexly or directly on the centre, adjust the pulmonary ventilation to the metabolic requirements, the primary function of the pulmonary vagus, in particular the augmentor influence, is so to adjust the rate of the rhythm that the wide fluctuations in the CO_2 and O_2 content of the blood which occur with each cycle in its absence should be prevented.

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THE OXYGEN AND CARBON DIOXIDE CONTENT OF THE BLOOD OF NORMAL AND PREGNANT DECEREBRATE CATS

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Preparatory to studying in the cat the foetal respiratory mechanism in relation to blood gases, few data concerning oxygen and carbon dioxide in cat's blood were found in the literature [Hering, 1867; Buckmaster & Gardner, 1910]. It was necessary to obtain new values under normal as well as the experimental conditions used in our foetal studies.

Methods

The micro-manometric method of Van Slyke & Neill [1924] for the combined determination of oxygen and carbon dioxide in blood was used. The procedure was that for 0·2 c.c. samples in the 50 c.c. apparatus. Small samples were analysed because this study was carried on in conjunction with foetal blood studies in which larger quantities were rarely obtainable, and comparable sets of values were desired. The blood was withdrawn under heavy mineral oil into tuberculin syringes through no. 27 needles containing minute amounts of heparin and was usually analysed within an hour. The blood was introduced below the gas-freeing solution in the pipette of the manometric apparatus directly from the needle of the syringe.

Most samples were taken from the femoral vessels, after dissection, but some were taken from the saphenous vein without dissection. The arterial and venous samples were not obtained simultaneously, from less than one to several minutes elapsing between their withdrawal.

Normal cats were strapped in resting position in a canvas hammock and local procaine infiltration of the femoral region was commonly

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practised. The pregnant animals were rendered decerebrate by ligating carotid and basilar arteries under ether, as previously described [Windle, Monnier & Steele, 1938]. One to several hours were allowed for recovery from the ether before blood was withdrawn.

RESULTS

The estimations of oxygen and carbon dioxide content of the blood of normal cats are presented in Table I and those of pregnant, experimental cats in Table II. Among the normal animals, the males showed higher average arterial oxygen content than the females. The non-excited females had higher average carbon dioxide content (above 40 vol. %) than those which were somewhat excited by the withdrawal of the blood.

TABLE I. Blood gases in normal cats

				Artery		Vein	
Cat	Sex	Anaesthetic	Excitement	CO_2 vol. %	O_2 vol. %	CO_2 vol. %	O_2 vol. %
A	♀	Nembutal	—	30·1	18·4	—	—
B	♀	,,	—	44·8	13·1	—	—
C	♀	None†	Yes	24·4*	14·3*	—	—
D	♀	None†	,,	27·2	15·9	29·7	11·5
E	♀	None	?	—	—	37·9	14·8
F	♀	,,	?	—	—	40·6	7·9
G	♀	Local	Yes	26·5*	19·2*	26·1	17·1
H	♀	,,	,,	34·1	13·1	—	—
I	♀	,,	,,	29·7	10·0	—	—
J	♀	,,	,,	33·3	13·2	42·9	10·1
K	♀	,,	No	42·5	15·6	47·2	11·1
L	♀	,,	,,	43·6	15·8	52·1	8·1
M	♀	,,	,,	40·5	15·4	45·6	12·8
N	♀	,,	,,	50·1	15·7	52·4	7·3
O	♀	,,	,,	41·4	13·8	51·7	8·4
P	♂	,,	Yes	36·2	21·9	39·6	16·8
Q	♂	,,	,,	33·6	16·8	36·9	14·7
R	♂	,,	,,	39·0	14·8	48·4	6·1
S	♂	,,	,,	35·5	16·0	46·8	9·5
T	♂	,,	,,	28·2*	18·7*	40·0	11·6
U	♂	,,	,,	26·0	15·9	37·1	10·0
V	♂	,,	,,	30·3	19·9	33·8	10·2
W	♂	,,	,,	31·3*	15·2*	46·8	10·2
X	♂	None	No	—	—	46·6	9·9
Average for females				36·0	14·9	42·6	10·9
Average for non-excited females				43·6	15·3	49·8	9·5
Average for males				32·5	17·4	41·8	11·0
Average of all cats				34·7	15·8	42·2	11·0

The dagger (†) indicates that the cat had been etherized about an hour before and the femoral vessel dissected at that time. The asterisk (*) indicates that two samples were analysed and averaged. Cats J and O are the same animal whose blood was analysed on two occasions 31 days apart.

TABLE II. Blood gases in pregnant experimental cats

	Foetuses					Artery		Vein	
Cat	Age days	Av. wt. g.	Placenta av. wt. g.	Respiration per min.	Decerebrate state	CO_2 vol. %	O_2 vol. %	CO_2 vol. %	O_2 vol. %
1	23	0·2	2·1	76	Active	33·3	19·4	40·0	4·5
2	28	0·5?	?	16	,,	36·9*	17·1*	—	—
3‡	35	4·0	9·9	CO_2	M. rig.	53·3	14·2	48·7	7·9
4	38	6·2	9·9	128	Active	18·8	13·2	29·1	8·9
5	38	6·7	9·0	44	M. rig.	28·1	16·6	37·5	8·7
6	?	17·0	15·0	28	Rig.	39·0	13·5	44·3	9·2
7	?	18·5	18·5	36 (deep)	M. rig.	15·4*	13·3*	31·8	7·0
8	46	17·0	11·0	Normal ?	Active	35·6	18·5	43·9	8·5
9	46	19·3	13·5	120–123	,,	9·4	17·9	11·3	14·4
10	46	20·0	15·0	Very rapid	,,	22·1*	14·5*	30·2*	7·7*
11	48	28·0	15·8	Irregular	M. rig.	36·3	15·9	35·9	12·1
12	?	29·0	17·0	24	Active	30·9	13·3	39·0	7·9
13	53	35·0	16·0	30	,,	23·9	13·1	26·2	6·2
15‡	?	36·5	15·1	CO_2	M. rig.	46·0	13·4	47·5	12·3
16‡	?	38·2	?	?	Not decereb.	31·8*	11·0*	46·2	3·3
17	?	42·9	23·1	32 (deep)	?	19·3	12·7	—	—
18	?	43·0	23·2	20	M. rig.	28·0	7·5	38·4	4·3
21	?	72·0	24·9	68 (deep)	,,	14·3	13·1	21·8	0·6
22	?	73·4	24·7	46	Active	30·0	13·4	34·5	7·9
23	?	74·0	22·0	28 (deep)	Rig.	29·2	11·0	36·8	10·7
24	?	75·0	16·5	40	?	29·6	11·2	34·6	4·7
26‡	?	97·0	21·5	CO_2	M. rig.	39·8	10·5	44·0	4·6
27	?	98·5	20·0	44	Rig.	26·4	12·2	35·2	9·3
28‡	?	105·0	24·7	Apnoea	?	37·4	6·4	37·1	2·7
29	65	111·4	23·9	Rapid	Active	30·0	13·4	34·0	7·6
30	63	115·0	26·6	?	?	25·8	10·9	35·7	10·9
31	?	118·4	25·1	Very rapid	Active	22·3	16·5	28·1	11·4
32(*a*)‡		?	?	Rapid	Not decereb.	21·6	8·7	33·1	1·6
32(*b*)‡				Normal	,,	41·7	9·5	47·1	2·9
32(*c*)‡				,,	,,	—	—	40·7	7·8
Averages of cats 23 to about 50 days gestation						27·8	15·7	34·3	8·9
Average of cats near term						25·3	12·3	32·5	7·4

The double dagger (‡) indicates experiments eliminated from the averages. In addition, cats 14, 19, 20 and 25 have been excluded from the present study; they were given spinal anaesthesia and results are not comparable with those presented. The asterisk (*) indicates that two samples were analysed and averaged. The femoral artery and femoral or saphenous veins were used in all except cat 29 in which carotid artery and femoral vein blood was taken. Under "decerebrate state" is indicated definite decerebrate rigidity (Rig.), marked rigidity (M. rig.) and transient ridigity with actively moving head and extremities (Active) at the time the blood samples were taken. Cat 16 was not decerebrated but had had the spinal cord sectioned a day before the experiment. Cat 32 was not decerebrated but blood was drawn from femoral artery and vein (*a*) during labour, (*b*) a day later, and (*c*) a week later. "CO_2" under "Respiration" indicates that the mother cat was breathing a mixture of 5 to 8 % CO_2 in O_2 at the time blood was drawn. "Apnoea" indicates an experiment in which the cat underwent temporary cessation of respiration due to change in its position while blood samples were being drawn.

The average arterial oxygen content of the decerebrate, pregnant cats, up to about the fiftieth day of pregnancy (pregnancy usually lasts 65–68 days in the cat) was similar to that of the normal animals. In animals beyond the fiftieth day of pregnancy, the average arterial oxygen content was reduced several volumes per cent below the normal. The averages for venous oxygen and carbon dioxide contents of these experimental animals were lower than those of the normal.

It is worthy of note that blood on standing for some time lost both oxygen and carbon dioxide to some extent by an apparently internal process [Huggett, 1926]. Of duplicate blood samples taken, one being analysed immediately and the other after several hours, the second showed its gaseous content reduced by 25–35 %. Mixing of the oil seal with the blood in samples standing for some time is an additional source of inaccuracy. These errors were overcome by analysing the samples as soon as possible after taking them.

COMMENT

Some of the normal animals were excited by the procedure of taking blood, with a consequent increase in respiratory activity. It is doubtful if this resulted in a significant increase in oxygen but it certainly brought about a decrease in the carbon dioxide content of the blood. Other cats were not excited but lay purring with respiration at its normal depth and frequency during the withdrawal of blood. Two, under light nembutal anaesthesia, displayed slightly slower and deeper respiration than normal. One might expect an increase in the carbon dioxide content of the blood from the less active respiratory exchange but little change in the oxygen content of the blood. It was hoped that an average of the entire series would give values within the normal physiological ranges of the two gases in artery and vein. Some individual variation between animals was to be expected. Although a rather wide fluctuation in the carbon dioxide content may well have been anticipated with varying respiratory activity, the normal range at rest is probably between 40–50 vol. %, as was found in animals definitely known to be unexcited, rather than the average 34·7 vol. % determined for the entire series. In the males the arterial oxygen content was higher than in the females. This is compatible with the higher erythrocyte count in the male [Scarborough, 1930].

The values for the cats in this study are higher than those of Hering [1867] and Buckmaster & Gardner [1910] made before methods of blood gas analysis had been brought to their present perfection. We have a larger series of determinations from which to arrive at average normal values.

The decerebrate, pregnant cats up to about the fiftieth day of gestation showed little difference in the arterial oxygen content from the normal. Beyond the fiftieth day of pregnancy a significant reduction in arterial oxygen content was observed. The results obtained in the unanaesthetized non-decerebrated cat no. 32 are of especial interest. The data suggest that decerebration had little if any effect in lowering the arterial oxygen content but that the reduced content in late pregnancy arose from physiological changes. It may be correlated with lowered erythrocyte count and tendency toward anaemia [Scarborough, 1930] and displacement of maternal oxygen dissociation curves to the right [Barcroft, Elliott, Flexner, Hall, Herkel, McCarthy, McClurkin and Talaat, 1934] in late pregnancy.

The lowered carbon dioxide content in the experimental animals seemed to be due principally to the hyperactivity, rigidity and respiratory changes produced by the decerebration. The reduced oxygen content of venous blood could be explained similarly. Rigidity resulted in increased muscle tonus, with a greater muscular consumption of oxygen and increase in the production of waste products of muscular activity. The reduced venous oxygen content thus arose from the increased use of oxygen by the hyperactive muscles. Increase in rate and depth of respiration could account for the lowering of the carbon dioxide content. An additional factor in the reduction of the carbon dioxide might well have been a decreased alkali reserve resulting from an attempt to neutralize the excess lactic acid produced by the hypertonic muscles.

CONCLUSIONS

1. Estimations of the oxygen and carbon dioxide content of normal non-pregnant and of decerebrate, pregnant cats' arterial and venous blood have been presented in tables.
2. The oxygen content of the arterial blood was higher in the male than the female cat.
3. Apparently there was a physiological reduction in oxygen content in the last two weeks of pregnancy.
4. The blood carbon dioxide fluctuated within wide limits in normal unoperated animals. This was correlated with degree of excitement and consequent increase in rate and depth of breathing while samples were being taken.
5. In blood of pregnant cats decerebrated by tying carotid and basilar arteries, carbon dioxide content was sometimes found to be below the lowest normal range. This appeared to be correlated with increased respiratory rate and depth (panting) and increased muscular activity and tonus.

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SOME CORRELATIONS BETWEEN RESPIRATORY MOVEMENTS AND BLOOD GASES IN CAT FOETUSES

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Foetal respiratory movements which occasionally can be palpated and observed visually in normal human subjects nearing term have excited interest for many years [Ahlfeld, 1891, 1905; Snyder & Rosenfeld, 1937], but the conditions under which they occur are not known. Attempts have been made to determine the relationship of carbon dioxide excess and oxygen want to their occurrence in experimental animals [Barcroft, 1935; Snyder & Rosenfeld, 1937; Windle, Monnier & Steele, 1938] by administering gas mixtures to the mother, but little has been done to correlate determinations of foetal blood gases with the presence or absence of these activities at the time of sampling. The present report concerns such correlations in the cat.

Methods

Pregnant cats were decerebrated by tying carotid and basilar arteries under ether and an hour or more was allowed for recovery before beginning experiments. The animals did not usually suffer lasting depression of respiration but, on the contrary, their breathing frequently became faster and deeper than normal. Blood pressure and body temperature were not greatly affected. In the instances in which breathing was slow, the cats were allowed to breathe a mixture of 5–8% carbon dioxide in oxygen by tracheal canula. They were placed in a constant temperature bath of Locke's solution where the uterus was brought forth and foetuses delivered through small incisions in least vascular parts without disturbing the zonary placentae. Foetal respiratory movements were studied while the specimens were submerged and with placental circulation intact. Later, some were allowed to breathe air.

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The quantity of blood obtainable from cat foetuses without seriously disturbing the total volume is limited. Consequently 0·2 c.c. samples were drawn under oil into tuberculin syringes with no. 27 needles containing minute amounts of heparin. They were analysed by the Van Slyke-Neill [1924] micro-manometric technique. Oxygen capacity was estimated by the Van Slyke-Neill method for 1 c.c. samples of blood after the foetal blood had been shaken repeatedly with air at atmospheric pressure in the froth chamber of the gas pump to bring about saturation. The principal error seemed to be introduced in measuring the quantity of blood.

RESULTS

Data on gas content of umbilical vein blood are presented in Table I. The carbon dioxide content of foetal blood was clearly an important factor in helping to determine the rate and depth of foetal respiratory movements. Thus, for example, foetus 4 of cat 15 managed to "breathe" at a rate of 28 per min. with only 3·7 vol. % oxygen, while other foetuses receiving as much or more oxygen were apnoeic. The difference lay in the carbon dioxide content which was high in the former and low in the others. In foetus 2 of cat 16, respiratory movements were weak and in foetus 4 of cat 15, strong and faster. More than 8 vol. % oxygen was present in both instances, but the former was poorer in carbon dioxide by 16·5 vol. %.

The presence or absence of respiratory movements could not be explained solely on the basis of carbon dioxide content. Foetuses receiving less than 3·7 vol. % oxygen in umbilical vein blood showed no respiratory movements or gasped infrequently, but most of those with greater amounts of oxygen did execute rhythmical respiratory movements. Relatively high concentration of carbon dioxide (40 % or more) in the absence of adequate oxygen in foetal blood did not lead to rhythmical respiratory movements but to arhythmical gasping. For example, the umbilical vein blood of foetuses 2 and 3 of cat 6 contained almost exactly the same carbon dioxide concentration, but only the specimen with higher oxygen executed rhythmical respirations. Similarly, foetus 3 of cat 17 lacked rhythmical activities when its blood was reduced (0·4 and 2·9 vol. % oxygen) but "breathed" at a rate of 14 per min. when the oxygen reached 5·1 vol. %, even though the concentration of carbon dioxide remained about 35 vol. %. It seemed that these differences in respiratory behaviour must have been related less to the carbon dioxide than to the increased oxygen content.

TABLE I. Carbon dioxide and oxygen content of foetal blood

Gasping or no respiration at sampling

Cat no.	Foetus No.	Foetus Wt. g.	Foetus Age days	Time min.	Umb. vein blood CO₂ vol. %	Umb. vein blood O₂ vol. %
6	2	18	?	6	53·3	1·1
8	2*	19	46	0	39·7	1·4
10	4	21	46	2	55·0	1·1
12	2	29	?	1	39·1	3·7
13	1	44	53	2	33·1	0·7
13	1	44	53	4	25·4	0·3
13	3*	28	53	0	31·5	4·8
17	3	38	?	¼	36·4	0·4
17	3	38	?	5	34·6	2·9
18	1	43	?	12	46·2	1·5
18	2	?	?	¼	51·6	1·6
22	1	73	?	5	36·4	4·5
23	1	77	?	2	41·2	1·6
24	4	84	?	4	24·0	2·8
26	1	103	?	1	53·8	0·6
26	1	103	?	3	47·2	3·6
27	1	104	?	3	24·5	2·5
27	1	104	?	5½	26·1	1·8
29	1	120	65	3	29·1	4·6
30	1	104	62	2½	33·3	1·3
31	1	118	?	1½	20·7	1·4
31	1	118	?	15	24·3	1·0
					Carot. art. CO₂	Carot. art. O₂
32	3	84	?	1	33·0	2·4
Average, apnoeic unborn foetuses					36·7	2·1
Average, active unborn foetuses					46·6	6·5

Rhythmical respiratory movements at sampling

Cat no.	Foetus No.	Foetus Wt. g.	Foetus Age days	Time min.	Umb. vein blood CO₂ vol. %	Umb. vein blood O₂ vol. %
6	3	17	?	½	53·8	4·0
11	3*	31	48	0	57·0	8·1
11	1	26	48	33	56·0	4·9
15	4	46	?	1	57·3	3·7
15	4	46	?	4	56·4	8·4
16	2	36	?	32	39·9	8·2
17	2	42	?	4	25·1	6·9
17	1	46	?	14	23·9	5·9
17	3	38	?	41	35·1	5·1
22	2	70	?	29	42·8	8·6
28	1	99	?	4	49·6	7·7
28	2	106	?	¼	56·5	8·1
28	2	106	?	12	59·4	5·3
29	2	104	65	53	43·6	7·8
30	3	125	62	?	42·8	4·6
					Carot. art. CO₂	Carot. art. O₂
19	1	52	57	20	39·3	14·9
19	1	52	57	90	32·7	5·0
19	2	46	57	30	36·1	6·9
23	2	71	?	60	36·6	13·2
23	2	71	?	105	27·6	6·9
26	1	103	?	37	38·8	12·0
26	1	103	?	65	54·3	6·5
28	1	99	?	165	57·7	6·6
29	3	115	65	19	43·3	9·7
30	3	125	—	1 day	43·4	13·0
32	4	?	—	1 day	51·6	13·4

The cats bear the same numbers in this and a preceding article [Steele & Windle, 1939]. Under the heading "time" is indicated the number of minutes elapsing between exposing the foetus and drawing the blood sample. In three foetuses marked with an asterisk (*), blood was drawn without removing the foetus from the uterus. Placental circulation was intact in all experiments in which blood was taken from umbilical veins. In those under the heading "carot. art." (carotid artery), the umbilical cords had been tied and the foetuses removed from the bath; "time" in these instances refers to minutes elapsed after tying the umbilical cords.

TABLE II. Oxygen capacity of foetal blood

Cat no.	Foetus No.	Foetus Wt. g.	O_2 capacity vol. %
6	1	18	11·0
12	2	29	15·5
18	1	43	11·6
23	1	77	16·9
23	2	71	14·8
26	2	90	15·6
27	2	100	14·8
28	2	106	15·9
32	3	84	11·5

A number of attempts were made to determine oxygen-carrying capacity of foetal blood, but the quantities of blood obtainable were so small that the figures in Table II may not be accurate and are probably too low. However, it becomes evident in comparing data in Tables I and II that rhythmical respiratory movements occurred in foetuses receiving blood no more than 40 or 50 % saturated with oxygen, and in others it could scarcely have been 30 %. Although it is yet to be proved what is the actual condition *in utero* for some cat foetuses in the last 2 weeks of gestation, 50 % saturation may not be a bad estimation, and for the following reason. In several experiments the umbilical vessels could be seen through small incisions in the uterus but without disturbing placenta and foetus. The veins were sometimes but not always brightly coloured. In one instance (foetus 3 of cat 11) a sample of blood was obtained without changing the scarlet colour of the vein. This blood contained 8·1 vol. % oxygen. Another time we were able to get blood with great dispatch at the moment of delivering foetus 2 from cat 28. It was brilliantly scarlet but began to darken as the last of the 0·2 c.c. sample entered the syringe. This contained 8·1 vol. % oxygen, about 50 % saturated. Both foetuses were executing rhythmical respiratory movements at rapid rates, due in part perhaps to the fact that the mother cats were breathing 5–8 % carbon dioxide in oxygen at the time and the foetal blood contained large amounts of carbon dioxide.

Some foetuses were allowed to be born, i.e. their umbilical cords were tied and they were brought out of the bath. Blood, obtained without anaesthesia from the carotid artery, was analysed after various periods of air breathing. In two 1-day-old kittens it contained quantities of carbon dioxide comparable with normal unexcited adult cats (Steele & Windle, 1939) and the oxygen was within normal range. Several, having breathed for shorter periods, gave similar oxygen values but the carbon dioxide was lower. The arterial blood of others was low in oxygen and some of the kittens showed less oxygen an hour or more after delivery than they had earlier. Perhaps they were not yet prepared to cope with air-breathing successfully. The data appear on the right side of Table I.

Apnoeic unborn foetuses receiving blood low in carbon dioxide were induced to gasp and start breathing air by pinching the umbilical cord. This was illustrated many times, and even in specimens whose umbilical vein blood proved to contain so little oxygen that further oxygen deficit could scarcely have been the only cause of the initial respiration. Umbilical vein blood was observed at the moment of natural delivery of cats 32 and 33. Its colour was dark and no different from blood of the umbilical artery. A sample of carotid artery blood of one kitten (no. 3 of cat 32) taken at birth (several gasps had occurred) gave low values for both oxygen and carbon dioxide. During labour, the mother cat was breathing rapidly, a fact which could account for the reduction of carbon dioxide. A litter-mate (no. 4), allowed to live 1 day, had high blood values for both.

It has been shown that maternal blood fluctuated within wide limits in respect to its content of carbon dioxide under varying conditions of excitement and rapid breathing [Steele & Windle, 1939]. In the present experiments in which the mother cats were breathing normally, slowly or in which oxygen and carbon dioxide mixtures were being inhaled, the foetal blood contained 40 vol. % or more carbon dioxide. But in the instances in which the cats were breathing faster than normally and were active or showed marked rigidity at the time foetal blood samples were taken, the carbon dioxide was lower. These differences in maternal conditions were correlated with variations in foetal activity.

Under experimental conditions, foetal respiratory movements varied greatly. Some specimens had rather long periods of activity during which the respiratory rate was 14–32 a minute and often deep. Others, when the mother cats were breathing oxygen containing carbon dioxide, executed short sequences of respiratory movements at rates of 42–76 or even 135 per min. The highest rates were observed soon after the gas mixture had been withdrawn from the cats and may be interpreted as "rebound" phenomena after excessive dosing with carbon dioxide [Windle & Nelson, 1938]. Even in unanaesthetized, unoperated cats near term, continuous respiratory activity of the foetuses was not the rule. We were never able to palpate more than six or eight movements in sequence alternating with long periods of inactivity.

There appeared to be a relationship between pre-labour uterine contractions and the respiratory behaviour of the foetuses [Windle, Monnier & Steele, 1938]. In late foetal life, contractions of the uterus were accompanied by reddening of umbilical vein blood which imparted a better colour to the foetuses themselves. Such blood contained more oxygen than it had during uterine relaxation. In Table I, the two values for specimen 4 of cat 15 and for foetus 2 of cat 28 were obtained from blood taken at different stages of uterine activity. Other differences seen in Table I, such as that between the two foetuses of cat 6, were related to the same phenomenon. The differences in foetal respiratory activities were evidently correlated with these changes in oxygenation.

COMMENT

The present studies, together with those of Eastman [1930] and Haselhorst & Stromberger [1930] in the human and Barcroft [1936] and Barcroft, Elliott, Flexner, Hall, Herkel, McCarthy, McClurkin & Talaat [1934] in the goat and sheep, indicate that umbilical vein blood is poorly saturated with oxygen toward the end of gestation (last quarter in the cat). Although 50% saturation or less may be the usual condition, not all investigators have obtained such low values and considerable variation has been the rule. Roos & Romijn [1938], who found the umbilical vein blood of one calf 90% saturated at 8½ months, obtained lower figures (47–75%) in four other 7–8 month foetuses. Barcroft & Mason [1938] have obtained a few high values in the sheep. Goldbloom & Gottlieb [1930] and Bidone [1931] reported high umbilical vein oxygen in human foetuses at birth.

We do not know whether foetal respiratory movements would manifest themselves in the absence of some degree of foetal anoxaemia (lowered oxygen and increased carbon dioxide) or not. Foetal anoxaemia was encountered in every experiment in the late foetal life of the cat. Earlier in gestation, the foetuses of decerebrated cats were apnoeic [Windle *et al.* 1938], and carbon dioxide inhaled by the mothers caused respiratory movements to start in these foetuses. No blood gas analyses could be obtained in these small specimens but perhaps, like the goat and sheep [Barcroft, 1936; Barcroft *et al.* 1934], the umbilical vein blood would have been found more nearly saturated with oxygen throughout the middle of the gestation period than towards its end. In view of the present experiments, in which it was observed that the faster respiratory movements were associated with the higher oxygen estimations, it is doubtful if the apnoea of younger foetuses was due entirely to high oxygen saturation. The part played by carbon dioxide must be taken into account.

A deficit of carbon dioxide seems to lead to apnoea in rabbit foetuses [Snyder & Rosenfeld, 1937], but what constitutes a normal level of this gas in foetal blood? The arterial blood of non-pregnant, unoperated, unanaesthetized female cats fluctuated widely (25–50 vol. %) in carbon dioxide content [Steele & Windle, 1939] depending in part upon the degree of excitement and rapidity of breathing while samples were being drawn. The average value was 36 vol. % carbon dioxide. Under our experimental conditions, blood of the foetuses showed similar fluctuations. The average carbon dioxide content of foetuses lacking respiratory movements or showing only occasional gasps was 36·7 vol. % and for those executing rhythmical respiratory movements, 46·6 vol. %. Others [Eastman, Geiling & De Lawder, 1933] observed that the carbon dioxide tension of human umbilical vein blood varied within wide limits. Keys [1934] reported that foetal goat blood had a higher carbon dioxide capacity than the maternal blood due to a greater alkali reserve. The data in these and other articles [Roos & Romijn, 1938; Bidone, 1931; Haselhorst & Stromberger, 1930; Kellogg, 1930] dealing with carbon dioxide content of umbilical vein blood in late pregnancy indicate that this gas is not unusually low according to adult standards but only relatively so, perhaps in respect to the foetal carrying capacity.

In some of the present experiments it would seem that conditions simulated asphyxia neonatorum. This is suggested by the very low content for both oxygen and carbon dioxide sometimes encountered in umbilical vein blood of the cat foetuses lacking respiratory efforts. Very rapid breathing of some of the mother cats together with probable excessive lactic acid formation during the activity and rigidity of the decerebrate state may have brought about the reduction of the carbon dioxide level. The blood gas analyses resemble those obtained in the human foetus in a state of asphyxia at birth [Eastman, 1932; Wilson, Torrey & Johnson, 1937].

The part played by the uterus itself in regulating the oxygen content of umbilical vein blood has received little attention previously. In the experiments in cat foetuses, alternate lightening and darkening of umbilical vein blood with uterine contractions was a very striking phenomenon. Here is a mechanism which, if operating under normal as well as experimental conditions, can well serve to alleviate periodically a normally existing state of partial anoxaemia in the foetus as birth approaches. The intermittent character of intra-uterine respiratory and other somatic foetal movements seen in normal unanaesthetized subjects may be correlated with this uterine activity.

CONCLUSIONS

1. The occurrence during the last quarter of foetal life of rhythmical respiratory movements in cat foetuses whose placental circulation was intact was related both to the oxygen and carbon dioxide content of umbilical vein blood.

2. The umbilical vein blood was at best little more than 50% saturated with oxygen in the last foetal quarter.

3. When the oxygen and carbon dioxide were very low (average 2·1 and 36·7 vol. %) no respiratory movements occurred.

4. Rhythmical respiratory movements were observed in foetuses receiving blood containing greater amounts of these gases (average 6·5 and 46·6 vol. %).

5. Arhythmical deep gasps like those initiating air breathing at birth were encountered in foetuses receiving blood very poor in oxygen but high in carbon dioxide.

6. The fluctuation of foetal blood within wide limits in respect to both oxygen and carbon dioxide content was apparently due to such factors as maternal respiratory rate and depth, muscular work done by the maternal animals and the contraction and relaxation of the uterine muscle. The uterine contractions were accompanied by intermittent improvement in oxygen content of umbilical vein blood.

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THE BLOOD VOLUME IN LAMBS

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Though estimations exist of the haemoglobin value of the blood in new-born and growing infants and the blood volume and plasma volume in the first 21 years of life in human beings [Seckel, 1936], no complete survey has been made of the blood volume, total haemoglobin content, plasma and corpuscle volumes, etc., in the young of any mammalian species from the time of birth onwards.

The present paper gives data on these subjects. Lambs were used as experimental animals. The haemoglobin was determined by Haldane's and Van Slyke's methods and the corpuscle, plasma and blood volumes by the haematocrit and the Congo red injection method. Approximately 0·7 c.c. of a 2% solution of Congo red per kg. of lamb was injected into the jugular vein, which was exposed by a skin incision under a local anaesthetic; there was therefore no doubt that the determined amount of dye went into the vein and nowhere else. The interval between the injection of the dye and the withdrawal of the subsequent test sample was 5 min. The samples were all satisfactory as regards the absence of haemolysis. Five lambs, all born naturally, were observed:

Serial no.*	Date of birth (1937)	Foetal age at birth	Haemoglobin value of mother c.c. O_2/100 c.c. blood	
188	16 March	144 days		
225	16 June	141 days	19·1 H.† 19·0 V.	5 May
244	19 May	148 days	13·6 H.	17 May
245	22 March	—	15·3 H. 14·7 V.	23 March
246	21 March	—	13·7 H. 13·5 V.	20 March

* These numbers are the same as those of the mothers in the series observed at Cambridge.

† H. = haemoglobinometric; V. = Van Slyke.

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[1] Rockefeller Fellow.

Fig. 1 shows the growth of lambs 188, 225, 244, 245, and 246. Of these, two, namely 188 and 246, may be regarded as normal during the first 6 months of their lives. Up to that point the growth curves run much the same course. Apart from a little irregularity at the commencement of the curve the absolute increment of weight is very uniform and is just over 1 kg. per week. In contradistinction to these two there was one lamb (245), whose weight is also shown in Fig. 1, which lost its mother on the 24th day of its life: it was then bottle fed on cow's milk. Up to that point it was a little larger than the others and was growing at the same rate. From the time it lost its mother till the 60th day when it was entirely living on grass—i.e. for just under 4 weeks—its weight remained almost stationary.

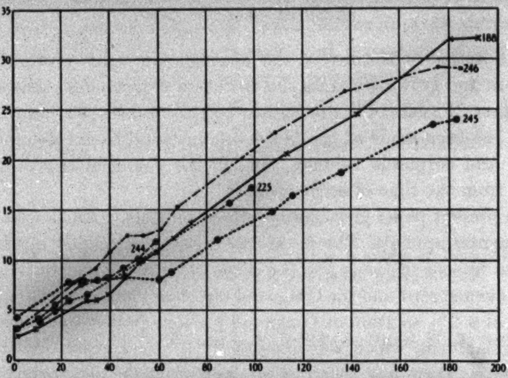

Fig. 1. Growth curves of lambs: ordinates, weight in kg.; abscissae, age in days. The numbers are the serial numbers of the mothers which are also used to denote the lambs.

Had 245 grown at the normal rate it would, when 60 days old, have been 4 kg. heavier than it was on the 60th day, i.e. it would have weighed about 12 kg.: actually it weighed 8·2 kg. It is not possible to state the cause of this failure to grow, though it opens up an interesting point for further research. One obvious possibility was dietetic insufficiency, another that the lamb suffered in a less degree from the disease from which its mother succumbed. Up to the end of the sixth month (183 days), when my observation ceased, this lamb had never made up the 4 kg. on its fellows 188 and 246. They weighed respectively 31·8 kg. (181 days) and 29·1 kg. (176 days), whilst 245 only weighed 24·2 kg. Its rate of growth, however, from the 60th to the 183rd day was not very different (a little slower only) from that of the normals.

Lambs 225 and 244 also dropped out of the category of normals. The mother of 244 died when it was 53 days old; that of 225 wasted and was

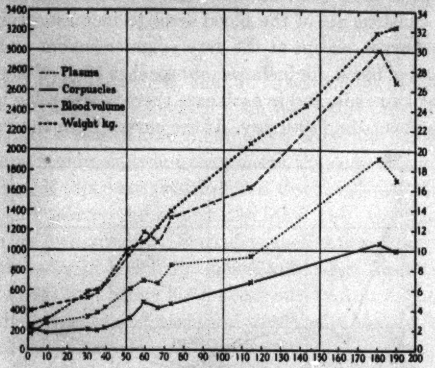

Fig. 2 *a*.

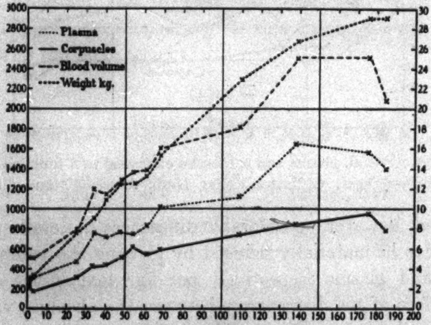

Fig. 2 *b*.

Figs. 2*a* and *b*. Left-hand ordinates, volumes of blood, plasma and corpuscles in c.c. Right-hand ordinates, weight in kg. Abscissae, time in days. (*a*) no. 188, (*b*) no. 246.

killed when it was 87 days old. Neither lamb showed more than a trifling falling off in the rate of growth. This rather suggests that the growth deficiency in 245 was dietetic, for the older lambs are naturally dependent upon their mothers to a much less degree. On the other hand, it is not clear what the maternal trouble was in any of the three cases; it may have been different in 245 from the others.

Figs. 2*a* and *b* represent the body weight, the blood volume, the plasma volume and the corpuscle volume of lambs 188 and 246 over the first 6 months of their lives. The two figures present a general similarity in that the constituents of the blood tend to increase over the general range of the curves, except at the very commencement and at the very end. The mass of blood, for instance, approaches 10% of the body weight except at the two ends, and in each case there is also a lag in the middle of the curve about the 110th day. If the curves from the two lambs are superimposed, however, considerable differences in detail appear. These differences can be materially reduced by plotting the volumes of blood, corpuscles and plasma respectively per kg. against the age (Fig. 3). Much therefore of the discrepancy between the absolute values for the two animals is bound up with the fact that 246 grew more rapidly at first and sooner reached a steady weight, and finally was lighter than 188. It would seem therefore that in normal animals the blood volume is related to the natural conditions of growth.

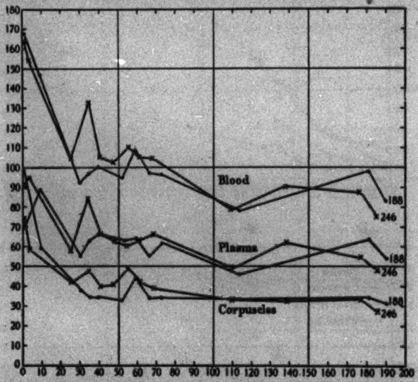

Fig. 3. Volumes of blood, plasma and corpuscles expressed as a function of body weight at different ages. Ordinates, c.c./kg. lamb. Abscissae, time in days.

Let us now consider some of the individual points brought out in Fig. 3. Are all lambs born with the same amount of blood per kg.? Table I gives the data from the five lambs on the first day. Of these 245 was a very large lamb and its blood volume, as compared with those of other

TABLE I

Lamb	Weight	Blood volume c.c.	Plasma volume c.c.	Corpuscle volume c.c.	Blood volume c.c./kg.	Plasma volume c.c./kg.	Corpuscle volume c.c./kg.
188	2·40	404	168	236	168	70	98
241	3·52	567	284	283	161	81	80
245	4·31	612	296	316	142	69	73
246	3·07	514	282	232	168	92	76
244	3·05	539	170	369	176	56	121

lambs, was not up to its weight, but the blood volume of the other four, 168 ± 8 c.c./kg., varied only about 5% on each side of the average. This, unless accidental, is remarkable in two ways. First, the blood volume per kilogram is much more constant than either the corpuscle volume or the plasma volume; if this were established it would indicate that the blood volume was not merely the sum of the other two, but was a quantity regulated on its own account. Secondly, such constancy is remarkable, in view of the way in which the blood volume at birth is arrived at. I have pointed out that before birth about one-quarter to one-third of the blood is in the placenta and umbilical vessels and that on birth a great proportion of this is expelled into the foetus.

The second notable feature in Fig. 3 is the reduction in the blood volume per kilogram and in both its constituents during the first 3 weeks of life. The question arises: Is this diminution absolute or is it only relative to the body weight?

TABLE II

Lamb	Day	Weight kg.	Blood volume c.c.	Plasma volume c.c.	Corpuscle volume c.c.	Blood volume c.c./kg.	Plasma volume c.c./kg.	Corpuscle volume c.c./kg.
188	1	2·40	404	168	236	168	70	98
	9	3·14	466	281	185	147	89	59
	30	5·8	533	321	212	92	55	37
244	1	3·05	539	170	369	176	56	121
	10	4·3	548	265	283	128	62	66
	17	5·5	647	370	277	117	67	50
	28	7·65	807	469	338	105	61	44
245	1	4·31	612	296	316	142	69	73
	2	4·7	656	369	287	140	78	61
	23	7·8	1050	528	522	135	68	67
246	1	3·07	514	282	232	168	92	76
	3	3·35	517	318	199	154	95	59
	25	7·7	800	479	321	104	62	42

Table II shows that the absolute blood volume does not decrease; there may be but little increase during the first few days, but the corpuscle volume undergoes considerable absolute diminution in every case. In lambs 188 and 244 the corpuscle volume at the end of 4 weeks was not so great as on the first day. Naturally, therefore, taking the findings about the blood and corpuscle volumes together, the haematocrit readings should fall in the period immediately after birth, as indeed they do.

Haematocrit readings

Lamb 188	Age in days	1	9	30	
	Haematocrit	58	40	40	
	Oxygen capacity	21·3	15·2	16·5	
Lamb 244	Age in days	1	10	17	28
	Haematocrit	69	52	43	43
	Oxygen capacity	24·0	21·1	16·6	15·1
Lamb 245	Age in days	1	2	23	
	Haematocrit	52	44	50	
	Oxygen capacity	19·4	18·0	19	
Lamb 246	Age in days	1	3	25	
	Haematocrit	46	39	39	
	Oxygen capacity	16·4	14·6	16·7	

Evidently at first more corpuscles are destroyed than formed, but the quotient of the oxygen capacity divided by the haematocrit reading does not show a difference large or constant enough to be significant.

The next point of interest is at about 35–40 days, where there is a discrepancy—and indeed the only discrepancy—between lambs 188 and 246. The former presents a peak at that point. At present the existence of this peak must be left open. The simplest explanation and perhaps the correct one is that of experimental error, though the observation commands as much respect as any other in the series. On the other hand, there is a similar peak in the curve of 244, but not of 225. In the case of 225, however, we have no actual determination between the 35th and 39th day. Lamb 244 could not, however, be regarded as normal, first, because between the 39th and the 45th day (namely just at the time under discussion) it suddenly became very anaemic; its oxygen capacity dropped from 13·5 to 8·75 c.c. O_2/100 c.c. of blood, and whether the previous peak had to do with this onset of anaemia it is impossible to say; but there was no such change in 246. Secondly, at the 52nd day it lost its mother, a fact which may not have been unconnected with its own nutrition. It seems then that there may be a critical point in the blood volume about the 35th–40th day, the establishment of which must depend upon future research.

The rest of the curves shown in Fig. 3 can be described very briefly. All the constituents remain at the level which they reached at the end of the 3rd week, or slightly above it till about the end of the 9th week. There is then a fall in the corpuscle volume, followed by a fall in the plasma volume. After that the corpuscle volume in relation to the body weight remains remarkably constant, falling a little at the end of 6 months. The plasma and blood volumes relatively to the body weight show a fall at about 15 weeks and a rise, followed by a fall, later.

Having treated of two normal lambs, and two others which for a time were not very abnormal, I think it is worth while to record the data of a lamb (245) which was abnormal in two ways. In the first place, it was very large as compared with others from the same flock, the mothers in which weighed as a rule rather under than over 30 kg.; and in the second place, it lost its mother when 24 days old and had to be bottle fed. The few lambs which we have had of over 4 kg. have in most cases been associated with early fatality either of the lamb or the mother. In at least one case the mother died before giving birth to the lamb, in another it was moribund, and in a third the lamb was dead when found in the morning, though it had breathed.

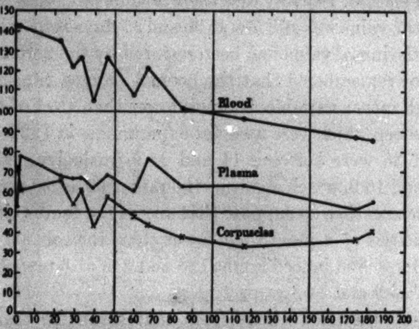

Fig. 4. Volumes of blood, plasma and corpuscles per kg., lamb 245: scales as in Fig. 3.

Lamb 245 was born not only with a smaller blood volume per kilogram than the normal but even more markedly with a smaller plasma volume, but as these did not undergo the usual degree of diminution during the first days they were not abnormal at the end of the 3rd week (Figs. 3, 4); indeed by the 60th day, when the lamb fed entirely on grass, the blood volume and plasma volume relatively to the body weight were on the high side, a fact rather associated with lightness of body than excess of blood. The most notable fact about the lamb was that from the 23rd to the 60th day neither the absolute volumes of the corpuscles, the plasma, nor the total blood, increased; in fact, they diminished.

OXYGEN CAPACITY OF BLOOD

The following were the oxygen capacities during the first month:

Lamb 188	Age in days	1	9	30	
	Oxygen capacity	21·3	15·2	16·5	
Lamb 233	Age in days	1	11	17	23
	Oxygen capacity	—	15·1	14·2	13·7
Lamb 244	Age in days	1	10	17	
	Oxygen capacity	24·0	21·1	16·6	
Lamb 245	Age in days	1	2	23	
	Oxygen capacity	19·4	18·0	19·0	
Lamb 246	Age in days	1	3	25	
	Oxygen capacity	16·4	14·6	16·7	

An interesting point about the above figures is that they do not present any common picture. In every case there is some fall, but whereas in lambs 188 and 244 the value was still low at 30 and 17 days respectively, in lambs 245 and 246 the initial value had been restored by the 23rd and 25th days.

It must be remembered that the normal oxygen capacity of sheeps' blood, though rather variable, is much lower than that of human blood; thus of 104 sheep which were used for experiments in 1937–8, the oxygen capacities of 36 were between 14 and 14·9 (inclusive), whilst 88 were between 13 and 16·9, which confirms the rather meagre text-book figures. There is no reason then to suppose that any of the lambs cited above had oxygen capacities at a month old lower than they would have for the rest of their lives, and indeed lambs 188 and 246 at 6 months had oxygen capacities of 13·9 and 14·3 respectively.

The picture therefore differs from that given for man by Josephs [1936], who has collected the records of a number of observers. These show a fall shortly after birth of about one-half to one-third of the natal value, but what is more important perhaps, the fall is to about one-third to one-half of the adult value.

THE TOTAL HAEMOGLOBIN IN CIRCULATION

On the assumption that 1 g. of haemoglobin is equivalent to 1·34 c.c. of oxygen, it is possible from the volume of circulating blood and the oxygen capacity to calculate the mass of haemoglobin in circulation. This for 246 and 188 is shown in Fig. 5. The curves show no special features, except the drop in the total amount of haemoglobin in circulation within the first few days. This drop is not very great; so far as the figures go it is from 64 g. to 52 in lamb 188 and from 63 to 56 in lamb 246, which does not show much evidence of haemoglobin breakdown, and it is soon made good. The quicker rise in the case of 246 is in accordance with the quicker growth of the lamb (see Fig. 1).

Still, the question may arise, why should the placenta at birth squeeze the maximum of blood into the foetus [Barcroft & Gotsev, 1937] if a considerable portion of the haemoglobin is destroyed in the first few days of life? It seems impossible to answer this question at the present time, but certain considerations may be worth stating:

(1) Haemoglobin is only one of many substances in blood and something quite different may be that which is most required by the body.

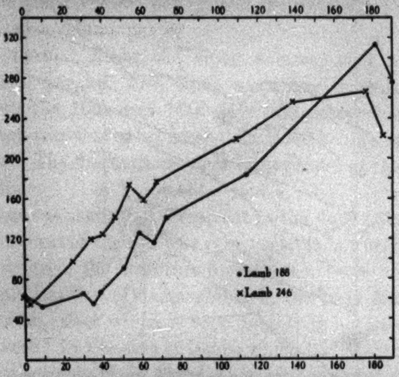

Fig. 5. Total circulating haemoglobin, lambs 188 and 246: ordinates, g.; abscissae, time in days.

(2) It is not certain that even if this blood were not expelled from the placenta into the foetus at birth, less would be destroyed afterwards. The destruction may not be the destruction of a surplus, but the surplus may be to forestall the destruction.

(3) It seems probable that even though the haemoglobin is in part destroyed, the iron may be saved and that this surplus-destruction mechanism may be one for obtaining a surplus of iron in the foetus as against the demand for future blood formation.

LAMBS WHICH HAVE LOST THEIR MOTHERS

The following three lambs lost their mothers at different stages: lamb 245 at 23 days, lamb 244 at 52 days and lamb 225 at 87 days. In every case the amount of haemoglobin in circulation received a check, and it is noteworthy that this check was much more pronounced than the check in growth of body weight. This point is brought out in Fig. 6. Thus lamb 245 did not contain appreciably more haemoglobin at 114 days than at

33, though nearly twice the weight. Having said so much it is difficult to say more, for in 244 the great loss of haemoglobin was before the mother died, and while it is true to say that the lamb contained no more haemoglobin at 120 days than at 40, it yet was making haemoglobin actively from the 52nd day onwards. The general course of events may be similar in the case of 225, where there is no observation between the 52nd and the 87th day. There may well have been a considerable drop in the curve of haemoglobin content during this period; at all events quite soon after the death of the mother the haemoglobin curve commenced to rise sharply. I am indebted to Dr Fraser for the suggestion that the mothers of 225 and 245 ceased giving milk some time before they died, with consequent worm infection of and anaemia in the lamb.

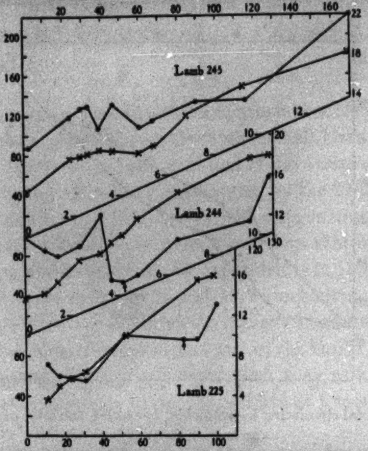

Fig. 6. Total circulating haemoglobin, lambs 225, 244 and 245. (•) left-hand ordinates, g. Body weight, (×) kg.; right-hand ordinates, kg. Abscissae, time in days. The arrows indicate the days on which the lambs lost their mothers.

Summary

1. Two normal lambs grew for the first 6 months at a uniform rate of about 1 kg./week. Two lambs, which lost their mothers at 53 and 87 days respectively, scarcely suffered in growth. One lamb, which lost its mother at 23 days, put on no more weight till 60 days, after which it grew at a normal rate but it did not make up the lost ground.

2. The blood volume at birth in the case of 4 lambs was 169 ± 8 c.c./kg. The fifth, a very large lamb, had a blood volume 142 c.c./kg. These figures show greater constancy than either the corpuscle volume or the plasma volume.

3. The blood volume in all cases increased from birth onwards, though in some cases slowly at first. This was due to an increase in the plasma volume for the absolute corpuscle volume dropped in the first days, as did the blood volume per kg. and the corpuscle volume per kg., but not the plasma volume per kg.

4. In two normal lambs the blood volume, plasma volume and corpuscle volume per kg. with some secondary irregularities fell off rapidly between the 10th and 16th days and more slowly about the 120th day. They rose somewhat after that, there being a second summit about 180th day. The fluctuations were rather those of the plasma than of the corpuscles.

5. The oxygen capacity fell somewhat in the days following birth in all cases; the amount of the fall was very variable but on the whole was less than that given in the literature for babies.

6. The total quantity of haemoglobin in the body fell by a trifling amount after birth, after which it rose roughly in accordance with the weight of the lamb. In the case of lambs which lost their mothers at 53 and 87 days respectively there was a great set back in the haemoglobin formation which did not show in the weight curve. This appeared to occur rather before the death of the mother than after. These lambs could go straight on to grass, etc. In the case of lamb 245, which lost its mother at 23 days and had to be bottle fed up to 60 days, there was no appreciable increase of haemoglobin between the 29th and the 114th day, though the weight curve commenced to rise on the 60th day.

7. The possible significance of the expulsion of blood from the placenta to the foetus is discussed.

I should like to express my thanks to the Medical Research Council who have defrayed the expenses of the research and to the authorities of the Cambridge University Farm who have stocked and kept the sheep.

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A NOTE ON THE MANOMETRIC ESTIMATION OF OXYGEN IN SMALL SAMPLES OF BLOOD

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From the Physiological Laboratory, Cambridge

(*Received* 6 *October* 1938)

When the initial volume of blood available for analysis is small, transfer to the Van Slyke-Neill manometric gas machine is best performed with a pipette in which dead space and drainage error have been eliminated. The following procedure was used in experiments in which the total volume of blood withdrawn for analysis has been 0·2–0·5 ml. The pipette employed is more convenient than that described by Guest [1931], in that variable volumes of blood may be transferred.

Apparatus

The only special apparatus consists of a 2·0 ml. Van Slyke-Ostwald pipette in which the stem above the bulb has been replaced by a section of a serological pipette calibrated at 0·01 ml. intervals. That part of the pipette below the stopcock should contain about 0·25 ml. and the bore of the tip should be rather narrower than usual.

Procedure

The blood sample, withdrawn anaerobically, is aspirated into a small cylinder (10 × 20 mm.) containing a few milligrams of potassium oxalate under a thick layer of mineral oil, and gently stirred. A small volume of physiological saline is degassed in the Van Slyke-Neill apparatus and the pipette filled with this to any appropriate mark on the calibrated stem above the bulb. The wall of the stem above the fluid level is dried with a pipe cleaner. Ferricyanide solution is then degassed as usual for the subsequent oxygen analysis.

A rubber dropping bulb is collapsed and placed over the upper end of the pipette so that there is a negative pressure over the gas-free saline. The tip of the pipette is then pushed through the mineral oil into the blood which just previously has been stirred carefully. The stopcock is now turned so that the blood slowly rises into the pipette to any point up to the level of the stopcock. The cock is closed, the dropping bulb removed, the pipette stem wiped free of oil and the usual rubber tip attached. By careful manipulation very small samples may be withdrawn without aspirating the mineral oil. The volume of blood taken is measured by the change in level of the gas-free saline in the calibrated stem.

In transferring the blood into the Van Slyke-Neill apparatus at least two minutes should be taken to allow the column of blood to flow out of the stem, thus leaving little residue to be washed in by the saline. The latter is best admitted in small spurts until its level reaches the base of the bulb of the pipette. The quantities of ferricyanide solution employed and the details of the subsequent analysis are those recommended by Peters & Van Slyke [1931] for 0·5 ml. of blood. All pressures are read at the 0·5 ml. mark and the "C" correction is established by similar analysis of gas-free saline.

Calculation of the oxygen content may be made from the factors given for any volume of blood by correction for the actual volume analyzed. Should CO_2 be determined the factors for this gas are calculated on the basis of the volume of solution over which its pressure is measured.

RESULTS

The following table shows a comparison between analyses of sheep's blood at various oxygen saturations by the procedure described and by the usual technique with 2·0 ml. samples:

TABLE I. Comparison of analyses of sheep's blood

2 ml. samples vol. %	Samples of 0·1–0·2 ml. vol. %
17·82	17·69
14·50	14·55
9·66	9·77
9·12	9·04
7·31	7·20
7·00	6·94
4·03	4·15
3·94	4·04

The analyses by the micro-technique agree with those made upon the larger samples within a range of ± 0·12 vol. %.

SUMMARY

A modification of the micro-technique of the Van Slyke manometric technique is described; the analysis of 0·1–0·2 ml. of blood gives agreement with the larger sample to within ±0·12 vol. %.

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ON THE PROPERTIES OF SECRETIN

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The properties of secretin were reviewed by the author in 1934. Since then the new facts reported in this paper have been discovered.

Amino acid composition

A fractional enzymatic analysis of crystallized secretin [Ågren & Hammarsten, 1937] provided the opportunity to study quantitatively the constituent amino acids of this polypeptide-like substance. The content of the basic amino acids had previously been determined [Ågren, 1934]. A calculation from these figures (Table I, col. 2) has been made on the assumption that the molecular weight of secretin is 5000 (see Table I, col. 5), the figure obtained by Svedberg [Hammarsten, Ågren, Hammarsten & Wilander, 1933] using the ultracentrifuge.

Table I. The ratio of amino acids in secretin after hydrolysis

			g.mol. per 100 g. of secretin		
Amino acid 1	Weight 2 %	Mol. wt. 3	Found 4	Calculated* 5	Ratio 6
Arginine	6·60	174	0·038	0·040	2
Histidine	3·96	155	0·026	0·020	1
Lysine	8·9	146	0·061	0·060	3
Proline	5·29	115	0·045	0·040	2
Dibasic amino acids	6·39	140	0·045	0·040	2

* Base = 1 mol. of histidine per 1 mol. of secretin (mol. wt. 5000).

By combining the quantitative figures of the three basic amino acids in secretin (col. 2) with the respective molecular weights the number of gram molecules of each formed in the hydrolysis of 100 g. of secretin is obtained. The corresponding calculated values are given in col. 5. It may be seen that the molecular ratios for histidine, arginine and lysine bear the simple relation 1 : 2 : 3. The histidine value [Ågren, 1934] agrees fairly well with 1 molecule of this amino acid per molecule of secretin, thus giving the ratio 2 molecules of arginine and 3 molecules of lysine.

That proline or its oxy-derivative was present in secretin could be ascertained from the fact that the Van Slyke determinations after hydrolysis gave the value 54·68 % of the total nitrogen as mono-amino nitrogen, whilst a Kjeldahl determination of the same fraction gave 59·55 % [Ågren, 1934]. In the absence of tryptophan the difference, calculated as gram molecules per 100 g. of secretin, corresponds to 2 molecules of proline or oxy-proline (Table I).

Proline was isolated according to Dakin's method [Dakin, 1920]. The solution of the completely hydrolysed, crystallized secretin (1 g.) was precipitated with phosphotungstic acid, and after the separation of the basic amino acids the filtrate was freed from phosphotungstic acid according to Van Slyke's method. The solution was then extracted for 72 hr. with butyl alcohol under reduced pressure. The concentrated butyl alcohol filtrate was dissolved in absolute ethyl alcohol, the alcohol removed and the residue dissolved in water, treated with mercuric acetate and baryta. There was no amino nitrogen in the filtrate. With corrections for the loss in analysis there was a recovery from the filtrate of 38 mg. of solids with a nitrogen content of 12·0 % (calculated for proline 12·1 %). The amino acids accompanying proline in the butyl alcohol extract were treated with 90 % methyl alcohol. The filtrate was added to the extract obtained by continuing the butyl alcohol extraction for a further 72 hr. with propyl alcohol. The alcohol was evaporated in a vacuum. The residue was dissolved in water. Non-amino nitrogen could not be detected in this solution. Thus probably all the non-amino nitrogen is proline nitrogen, and 2 molecules of this acid are built into the secretin molecule.

The amide nitrogen in the hydrolysed secretin corresponded to 2 molecules of dibasic amino acids (Table I). These were isolated by the method of Jones & Moeller [1928]. The hydrolysate used in the identification of proline was employed for this purpose. The dibasic acids remained in the hydrolysate after the butyl alcohol extraction [Dakin, 1920]. They were precipitated as barium salts. The barium salts were decomposed with sulphuric acid, and after the removal of the sulphuric acid the concentrated solution was saturated with hydrochloric acid gas. This yielded 22 mg. of pure glutamic acid hydrochloride, which was identified by melting-point and nitrogen determinations (calculated for 1 molecule of glutamic acid per molecule of secretin 29 mg.).

The filtrate and washings from the glutamic acid hydrochloride preparation were freed from hydrochloric acid and evaporated to dryness. There was no acetic acid-soluble material present, which demonstrates the absence of hydroxyglutamic acid in secretin. The dry residue dissolved in water was boiled with copper carbonate, 30 mg. of a crystalline copper salt were formed and centrifuged. It contained 23·25% copper [Eisler, Rosdahl & Theorell, 1936] (calculated with 4½ molecules of water, 23·07%). The yield of pure aspartic acid was 23·1 mg. (calculated for 1 molecule of aspartic acid per molecule of secretin, 26·7 mg.). Thus secretin obviously contains 1 molecule of aspartic and 1 of glutamic acid.

The mean molecular weight of the ten identified amino acids is 146, which in turn leads to the value of 128 for the average weight of the amino acid residues. This accounts for one-fourth of the secretin molecule. Estimating the mean molecular weight of the amino acids formed by the complete hydrolysis of secretin as about 100, there must be about 50 amino acids in the secretin molecule.

STABILITY OF SECRETIN

The *p*H stability of secretin has previously been determined [Ågren, 1934]. Acid solutions of secretin (*p*H 2·0–6·0), which had been boiled 1–3 min., could be stored at +2° C. for about a week without any loss of activity. The storing of secretin solutions for longer periods has been carried out, after the hormone was used for routine intravenous injections on man [Ågren & Lagerlöf, 1936; Ågren, Lagerlöf & Berglund, 1936; Hammersten, Ågren & Lagerlöf, 1937].

The stability of secretin dissolved in several combinations of organic solvents was first tested. The best results were obtained with a glycerol solution (30 c.c. of glycerol diluted with water to 100 c.c.). Solutions of crystallized secretin or the secretin prepared for clinical use were stored at +2° C. Each contained 1200 cat units per c.c., this being the dose of secretin calculated for injection, when the body weight of man was 80 kg. The reaction of the solutions was acid because of the hydrolysis of the secretin chloride (*p*H 2·5). The secretin activity of the glycerol solutions was standardized monthly during half a year. The method for assaying the various solutions was that described by Ågren & Wilander [1932]. After 6 months the secretin activity was still constant, and the solutions were absolutely clear. For injection on man it was thought advisable to dilute 1 c.c. of the glycerol solution 1 : 5 or 1 : 10 with water, thus giving a 3% glycerol solution, which is about isotonic with the blood (calculated 2·78%). According to a personal communication from Dr

Lagerlöf at St Erik's Hospital, Stockholm, no ill effects were observed by injections on man of 5 c.c. of a 30% glycerol solution containing secretin.

When the glycerol solutions of secretin were neutralized to pH 5·0 or 7·4 a flocculent precipitate appeared instantly at pH 7·4 and after 2–3 days at pH 5·0. The precipitate at pH 7·4 contained 80–90% of the secretin activity. The pH-5-precipitate contained 20–30% of the total secretin activity and consisted mainly of unspecific proteins. By successive precipitation of impure secretin at pH 5·0 and 7·7 in 30% glycerol solution highly purified secretin preparations could be obtained. As previously shown [Ågren & Hammarsten, 1937] the dissociation of proteins and consequently their solubility are extensively diminished in glycerol solution. The purification of protein-like substances with specific activities, such as secretin, could be facilitated by isoelectric precipitation from glycerol solutions. The general usefulness of the method is obvious.

The stability of crystallized secretin stored in a dry state at room temperature seems to be remarkably high. Thus a preparation made in December 1933 had the same activity (250 cat units per mg.) when tested in August 1937.

THE PROTEOLYTIC ENZYMES IN HUMAN PANCREATIC JUICE

It has been shown that secretin stimulates the pancreas of man to a real secretion of enzymes [Ågren & Lagerlöf, 1936]. The proteolytic activity of the pancreatic juice in these earlier investigations was determined by measuring the digestion of casein only and nothing was stated about the different proteolytic enzymes present.

The detailed procedures for obtaining the pancreatic juice are described in previous papers [Ågren & Lagerlöf, 1936; Ågren *et al.* 1936]. It was important that the juice should be as free as possible from contaminating intestinal enzymes. The pancreatic secretion from the first period of 10 min. after the secretin injection (usually 50–60 c.c.) was rejected and the enzyme determinations were made on the juice collected during the following 40 min. The micro methods of Lindeström-Lang & Holter [1931, 1933] were used, the substrates being a 0·2 mol. solution of alanyl-glycyl-glycine (pH 7·4) in the determinations of aminopolypeptidase, a 0·2 mol. solution of glycyl-glycine (pH 7·4) in the determinations of dipeptidase, a 0·2 mol. solution of chloracetyl-tyrosine (pH 7·4) in the determinations of carboxypolypeptidase and a 4% solution of casein (pH 7·8) in the determinations of tryptic activity. Typical results obtained on four normal individuals are given in Table II.

TABLE II. The peptidase and tryptic activity per c.mm. of human pancreatic juice determined at 40° C. for 30 min.

Exp.	Aminopolypeptidase activity c.mm. $N/20$ HCl	Dipeptidase activity c.mm. $N/20$ HCl	Carboxypolypeptidase activity c.mm. $N/20$ HCl	Tryptic activity c.mm. $N/20$ CH_3COONH_4
1	0·010	0·011	1·14	0·67
2	0	0	0·86	1·36
3	0·023	0	0·98	1·14
4	0·011	0·012	1·24	1·47

The results show that there is about as much carboxypolypeptidase as tryptic activity in human pancreatic juice. Aminopolypeptidase and dipeptidase are not regularly present. The minimal traces of activity found in some instances might be due to a slight contamination with intestinal juice. It is a well-known fact that glycerol extracts of the pancreas contain large amounts of all three peptidases. On the other hand, by secretin stimulation only carboxypolypeptidase seemed to be regularly secreted from the gland. This result can be explained in several ways. The secretion of aminopolypeptidase and dipeptidase from the pancreas might be caused by vagus stimulation or it may be that these peptidases in the pancreas are intracellular enzymes, and the dipeptidase and aminopolypeptidase in the digestive juice as collected are secreted from the intestinal glands.

ACTION OF SECRETIN ON THE PANCREAS

It is important to know how secretin stimulates the activity of the pancreas, and in trying to solve this problem several investigations have been carried out [Babkin, 1928]. Secretin may act directly on the secreting cells or by way of the nervous system. Experiments performed with crystallized secretin have shown that the effect was not influenced by excluding different parts of the nervous system. Poisons such as *luminal*, *morphine*, *nicotine atropine* and *ergotamine* had no influence. When the gland was isolated from its nervous connexions crystallized secretin had the same effect as before. Thus after total extirpation of the solar plexus and the phenolization of the blood vessels there was no alteration in the rate of the secretion. Pilocarpine is the only nervous poison so far known which inhibits the secretin effect indirectly by spastic contraction of the ducts of the gland. Ergotamine inhibits this effect of pilocarpine [Eisler & Ågren, 1936].

On injecting solutions of potassium arsenite into the pancreatic arteries the following results were obtained: six cats in urethane narcosis with a body weight of 3·5 kg. were used. In all experiments 20 cat units of crystallized secretin were injected. The inhibitory effect of arsenic was determined as the decrease in the total amounts of bicarbonate in the pancreatic secretion after the injection of arsenic + secretin. The total amount of arsenic varied between 25 and 3 mg. of As_2O_3 and was given in the three isolated arterial blood vessels. 25 mg. of As_2O_3 gave complete inhibition of the secretin effect, 12·5 mg. of As_2O_3 decreased the effect to 30% of the starting value and 3 mg. of As_2O_3 to 70% of the same. Three hours after the injection of 12·5 mg. of As_2O_3, which gave the usual inhibitory effect, the secretin action was absolutely restored to the starting value.

It was determined, by direct inspection, that the whole gland in all experiments was perfused with the arsenic solution. The reversibility of the inhibitory effect proved that the injections of arsenic caused no severe injury. The evidence was confirmed by microscopical examination. Thus secretin stimulation of the pancreatic secretion was inhibited only by a poison with a general protoplasmatic effect, which is in accordance with the results obtained above and supports the assumption of the direct action of secretin on the glandular cells of the pancreas.

Summary

1. Proline, glutamic and aspartic acids have been isolated from the hydrolysate of crystallized secretin. It has been shown that the molecule of secretin contains 2 molecules of arginine and proline, 1 molecule of histidine, aspartic and glutamic acids and 3 molecules of lysine.

2. In a 30% glycerol solution stored at +2° C. the activity of secretin is constant for 6 months.

3. After secretin stimulation, the pancreatic secretion in man displays equal amounts of carboxypolypeptidase and tryptic activity. Aminopolypeptidase and dipeptidase are not regularly present.

4. Injections of arsenic solutions into the pancreatic arteries completely inhibited secretin action but after 3 hr. it was restored to normal.

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THE RELATION BETWEEN CONDUCTION VELOCITY AND THE ELECTRICAL RESISTANCE OUTSIDE A NERVE FIBRE

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Nervous transmission is generally believed to depend upon an electrical process, but the precise way in which one section of a nerve fibre activates another is still uncertain. According to the membrane theory, restimulation is brought about by the local electric currents which spread in advance of the active region. In the conventional form of the theory the current is assumed to flow in one direction along the core of the fibre and to return through the conducting fluid outside. If this view is correct, the velocity of transmission should vary with the electrical resistance outside a nerve fibre. Thus in Lillie's iron wire model, the velocity of propagation may be altered over a wide range by changing the volume and hence the total conductance of the fluid outside the wire [Lillie, 1924]. The experiments to be described in this paper were undertaken in order to decide whether the rate of propagation in a nerve fibre can be affected in a similar way. Single fibre preparations from crab or squid were employed, and the external resistance was changed by lifting the fibres out of sea water into a layer of aerated paraffin oil, or in the case of the squid fibres into an atmosphere of moist air. In sea water the fibres are shunted by a large volume of conducting fluid, but in oil or air they are surrounded by a thin film of saline, so that the external resistance would be high. This method of changing the external resistance has the advantage that the fibres are always in contact with a layer of sea water, so that their immediate environment remains unchanged throughout the experiment. Hence any changes in velocity produced by this treatment must be entirely due to the alteration in electrical resistance and cannot be attributed to chemical or ionic effects.

Experiments with crab axons

Single fibres were isolated from the limb nerve of *Carcinus maenas* by a method which was described in a previous paper [Hodgkin, 1938]. An account of the stimulating and recording apparatus was also given in this paper, so that they need not be described here. The arrangement for comparing conduction velocity in oil and in sea water is shown in Fig. 1. It consisted of four platinum electrodes (*B–E*), a fine glass hook (*G*) and two holders (*A*, *F*) which were made from fine-tipped forceps. When the dissection was complete, the electrode system was lowered into the sea water and one end of the fibre was gripped by the holder *A*. The other end was then seized with a needle and the intermediate stretch looped above the electrodes and beneath the glass hook.

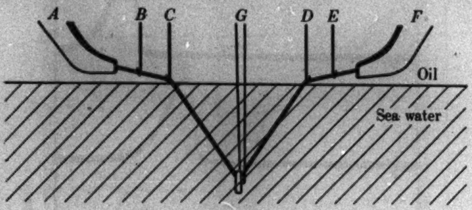

Fig. 1.

Finally, the free end of the fibre was gripped in the second holder *F*, and the glass hook was gently lowered until the fibre was taut. The whole system of holders and electrodes was firmly attached to a movable stage which could be raised or lowered by means of the screw adjustment on a Palmer stand. In order to measure the conduction velocity in sea water, the fibre was raised until the electrodes *C* and *D* were just clear of the interface. At this point both stimulating and recording leads were insulated by oil, but the greater part of the intermediate conduction distance was immersed in sea water. In order to determine the conduction velocity in oil it was only necessary to raise the electrode system until the whole of the fibre had passed through the interface.

The results of a typical experiment are illustrated by Fig. 2. In *A* the intermediate conduction stretch was immersed in sea water and in *B* it was raised into oil. This had a marked effect on the conduction rate, since the velocity was about 30 % greater in *A* than in *B*. The change was completely reversible, for the conduction rate returned to its original value when the fibre was replaced in sea water (*C*).

In order to make certain that the observed changes were not due to alterations in latency, the leads were arranged so that the action potential could be recorded before it entered the sea water as well as after. Measurements of this kind gave an entirely satisfactory result, for they showed that the latency did not change by more than 50 μsec., whereas the total conduction time might alter by as much as 1000 μsec.

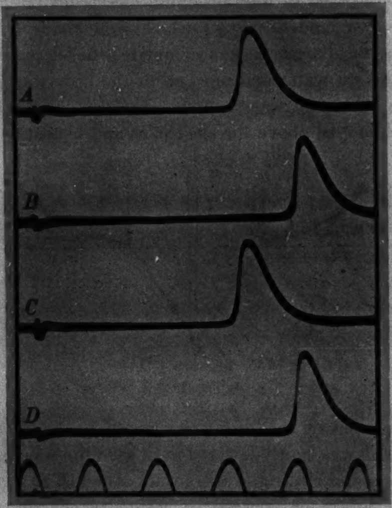

Fig. 2. *A* and *C*, action potential recorded with sea water covering 95 % of intermediate conduction distance, *B* and *D*; fibre completely immersed in oil. Conduction distance 13 mm. Time msec.

By lowering different fractions of the nerve fibre into sea water it was possible to show that the increase in conduction rate must occur to a uniform extent throughout the fibre. Fig. 3 illustrates the result of an experiment of this kind. In *A* the entire conduction distance was surrounded by oil, and in *B* 95 % of it was covered by sea water. In *C* one-third was raised into oil and two-thirds were left in sea water, whereas in *D* two-thirds were in oil and only the central third remained in sea water. Finally, in *E*, the entire conduction distance was again raised into oil. This experiment shows that the decrease in conduction time is roughly proportional to the length of nerve immersed in sea water, as it should be, if the effect depends upon a uniform increase in conduction velocity.

In some of the records in Fig. 3 the base-line is disturbed by small irregularities which occur between the application of the stimulus and the arrival of the action potential. These disturbances usually occur at the moment when the action potential crosses the oil-sea water interface or passes some other discontinuity on the nerve. They are to be regarded as artifacts and occur because the potential difference between the ends of the nerve fibre undergoes a sudden change at the moment when the action potential enters or leaves the sea water, thus producing a transient flow of current through the stray capacities between the stimulating and recording leads. Artifacts of this kind were not observed in similar experiments with the squid giant fibre, because this has a much lower resistance, so that effects due to stray capacity are relatively unimportant.

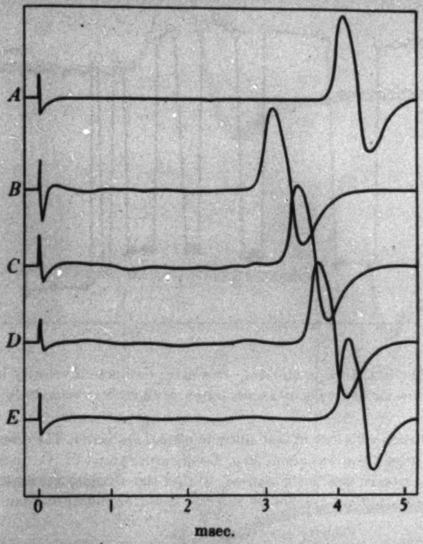

Fig. 3. *A*, *E*, fibre completely immersed in oil. *B*, 95 % of conduction stretch in sea water. *C*, 67 % in sea water. *D*, 33 % in sea water. Conduction distance 13 mm.

The process of transferring a fibre from oil to sea water occupied only a few seconds, so that a large number of observations could be made on one fibre. A series of measurements extending over a period of 30 min. is shown in Fig. 4, and provides a good demonstration of the speed and reversibility of the velocity change.

Table I summarizes the results of the experiments with crab fibres and shows that there is considerable variation in the magnitude of the velocity change. This probably depends upon differences in the amount of connective tissue left on the fibres, since any adherent tissue must lessen the external resistance in oil and so facilitate the propagation of the action potential.

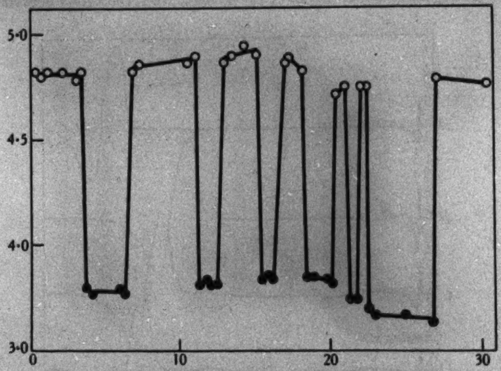

Fig. 4. Abscissa: time in minutes. Ordinate: conduction velocity in m./sec. Hollow circles: velocity in sea water. Full circles: velocity in oil.

TABLE I. Conduction velocities of crab fibres in oil and sea water. The diameter of the axis cylinder of these fibres was about 30 μ. Temperature about 21° C. In certain cases the conduction distance was not measured, so that the absolute values for velocity were unknown, although the percentage increase could be obtained from the conduction times.

Velocity in oil m./sec.	Velocity in sea water m./sec.	Percentage increase
—	—	35
—	—	33
2·8	3·9	40
4·1	5·3	30
3·8	4·9	28
—	—	33
3·4	4·5	33
—	—	25
3·6	4·1	14
3·4	4·0	18
3·5	4·0	15
3·0	3·9	30
2·6	3·1	20
4·4	5·5	26
4·0	5·2	30
3·5	4·5	29

Experiments with the squid giant axon

The giant fibres used in this work were obtained from the hindmost stellar nerve of *Loligo pealii* [Young, 1938] by the method described by Cole & Curtis [1939].

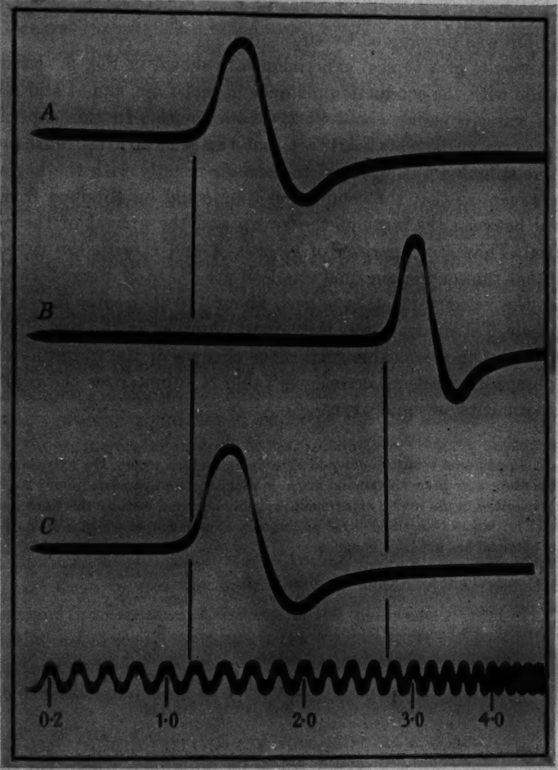

Fig. 5. *A*, *C*, velocity with 95 % of conduction distance in sea water. *B*, whole fibre in moist air. Time base 5000 cycles. The figures give the time in msec. after the shock. Note that the sweep is exponential. Conduction distance 26 mm.

The stimulating and recording apparatus were slightly different from those used in the previous experiments, but need no special description.

The electrode system was similar in principle to that in Fig. 1, except that the leads were sealed into a wax cell and the axon was immersed in sea water by raising the interface instead of by lowering the electrodes. The giant fibre survived quite well in an atmosphere of moist air, so that it was unnecessary to use oil as an insulating medium and the external resistance could be changed by lowering the sea water until the whole fibre was surrounded by air.

The results of a typical experiment are shown in Fig. 5. Record *B* was made with the conduction distance in moist air, and *A* and *C* with the sea water covering about 95 % of its length. In this experiment there was no visible shock artifact, but this was of little consequence, since the stimulus was arranged to coincide exactly with the beginning of the sweep, so that the conduction time could be obtained from the interval between the start of the sweep and the arrival of the action potential. Thus a comparison of records *A* and *C* with the time base shows that the conduction time was 1·15 msec.[1] In *B* it was 2·75 msec., so that the velocity must have been about 140 % greater in sea water than in air. This increase was the largest observed in the present work, but it was by no means abnormal, since changes of 100 % or more have been observed on several occasions. Table II summarizes the results of the experiments on squid axons.

Table II. Conduction velocities of squid fibres in air and sea water. The axis cylinders of these fibres were between 480 and 520 μ. in diameter. Temperature, about 20° C. The low velocities in the second experiment probably occurred because this fibre was kept for 11 hr. before being used. One preliminary experiment in which a 60 % increase was obtained has not been included.

Velocity in air m./sec.	Velocity in sea water m./sec.	Percentage increase
11·7	25·0	114
(5·6)	(10·6)	89
9·5	22·6	138
13·8	26·8	97
11·3	25·5	134

These large changes could only be obtained when the axons were very carefully cleaned from all loose connective tissue. A good illustration of this is afforded by an experiment in which measurements were made before the final cleaning of the fibre had been completed. Under these conditions the following values were obtained:

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[1] Conduction times were measured between the beginning of the shock and the moment when the action potential rose to 5 % of its maximum.

Velocity in sea water = 25·4 m./sec.
Velocity in air = 17·7 m./sec.
Increase = 44 %.

The fibre was then replaced in sea water for 3 hr. and as much connective tissue as possible removed from it. When this operation was complete the velocities were again measured and found to be as follows:

Velocity in sea water = 25·0 m./sec.
Velocity in air = 11·7 m./sec.
Increase = 114 %.

This experiment shows that the removal of connective tissue had no appreciable effect on the velocity in sea water, and indeed there is no reason why it should, since the resistance of a large volume of sea water is so low that the velocity would be limited only by the internal resistance of the axis cylinder. However, in air the adherent tissue plays an important part, since it lowers the external resistance and thus allows the action potential to propagate faster than it would in a cleanly dissected fibre. Similar considerations also account for the quantitative difference between crab and squid fibres, since the ratio of adherent material to axis cylinder is greater in crab fibres, so that the velocity change should be smaller than in the squid preparation.

VELOCITY IN NERVE TRUNK

Experiments with whole nerve trunks showed that the velocity of the giant axon *in situ* was approximately equal to the velocity of the same axon in a large volume of sea water. This result seems reasonable, since the resistance of the nerve trunk would be low compared with that of the axis cylinder, so that the electrical conditions would approach those of an isolated axon in sea water.

SIMILARITY IN THE ACTION OF OIL AND AIR

In most of the work on squid axons air was used as an insulating medium, but on two occasions oil was used, in order that its action might be compared with that of air. These experiments showed that both media produced approximately the same decrease in velocity, which is a satisfactory result since it indicates that the effect of paraffin oil is solely due to its properties as an insulator.

ALTERATION OF CONDUCTION VELOCITY BY METALLIC CONDUCTORS

A few experiments were made in order to discover whether the conduction velocity could be accelerated by means of a metallic short circuit. This was done by placing a grid of platinum strips between the stimulating and recording leads. These were sealed into the moist chamber and arranged so that they could be connected together by means of a mercury switch (Fig. 6). The fibre was mounted in position, and the conduction velocity measured with the switch first in one position and then in the other. This method of changing the external resistance has the advantage that the fibre itself remains in a constant environment and the only condition altered is the position of a switch outside the moist chamber. Measurements were made on three axons and in each case the conduction velocity was found to be greater when the metal strips were connected together (Table III). The effect was not large, but it was striking to watch on the oscillograph screen, since it took place within the fraction of a second required to move the switch. Table III shows that the change in velocity was much smaller than that observed in the air sea-water experiments. There are two good reasons for this difference. In the first place, the metal bridges are polarizable, so that

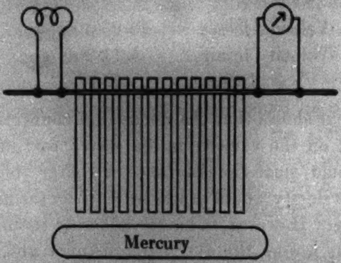

Fig. 6. Diagram of apparatus for short-circuiting giant fibre with metal conductors.

TABLE III. The velocities given are the average velocities between stimulating and recording leads. In the first case, the conduction distance was not measured.

Velocity with strips disconnected	—	14·7 m./sec.	12·5 m./sec.
Velocity with strips connected	—	17·8 m./sec.	14·3 m./sec.
Percentage increase	12	21	14

they offer an appreciable resistance to the action potential; and in the second place they only connect certain discrete points on one side of the fibre, instead of short-circuiting the entire surface as sea water does.

METAL BRIDGE EXPERIMENT

One preparation afforded an opportunity for observing a phenomenon which resembled that in the *Nitella* salt bridge experiment [Osterhout & Hill, 1930]. A fibre was left in the electrode chamber at the end of an experiment, and after a little time it was found that a spontaneous block had developed between two of the platinum strips. This was only effective when the strips were disconnected; if they were joined by the mercury contact, the action potential was able to traverse the injured region and so reach the recording leads. This condition did not last long, because the intensity of the block soon deepened to a point where it could no longer be relieved with the short circuit. There is no reason to doubt this effect in spite of its transient nature, for the result is an obvious corollary to the preceding experiments.

DISCUSSION

The results of the present research are very similar to those obtained by Auger [1933, 1936]. Auger measured the velocity of propagation in *Nitella*, and showed that it could be accelerated by covering the *Nitella* cell with a strip of moist filter paper. He considered that the increase in velocity could only be due to the decrease in external resistance, and concluded that his results constituted strong evidence for the local circuit theory. With the exception of this experiment on *Nitella*, all previous attempts to demonstrate a relation between conduction rate and electrical conductance have depended upon altering the chemical composition of the fluid bathing the tissue. Thus Pond [1921] immersed skeletal and cardiac muscle in solutions of low salt content and showed that this treatment led to a reduction in conduction velocity. This experiment is suggestive, but is not completely conclusive, because the observed change in velocity might have been due to an alteration in membrane excitability resulting from the decrease in salt content. This objection can be raised to any method which depends upon altering the constitution of the external medium. It does not apply to the present experiments, because in them the external resistance was changed by altering the volume and not the chemical constitution of the fluid outside the nerve fibre.

Summary

The following experiments show that the speed of propagation depends upon the electrical resistance outside a nerve fibre:

1. The conduction rate of an isolated crab fibre was 14–40 % faster in sea water than in oil. The external resistance was increased by oil, because the fibre was surrounded by only a very thin film of salt solution.
2. The velocity in the giant axons of the squid was 80–140 % faster in sea water than it was in air or oil.
3. A giant axon was laid on a series of metal strips which could be joined by a mercury contact. The velocity was accelerated by 12–21 % when the strips were connected together.

These results strengthen the local circuit theory of nervous transmission.

The work on squid axons was made possible by the kindness of Dr Cole and Dr Curtis who demonstrated the dissection of the giant fibre and allowed me to use their equipment at Wood's Hole. I wish to express my gratitude to them, and at the same time to Dr Gasser for assistance and hospitality in New York.

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THE OXYGEN IN THE CAROTID BLOOD AT BIRTH

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The present research arose from a desire to obtain a record of the alteration of environment of the brain, in respect of oxygen, at birth.

Method

Our experiments have for the most part been carried out by the continuous oxygen registration method of Kramer [1929] in which the oxygen saturation in the carotid blood of the foetus was measured by means of a photoelectric cell. This method depends upon the fact that oxyhaemoglobin transmits more light in the red end of the spectrum than reduced haemoglobin. A small unit is placed over the blood vessel in such a way that light from a micro-lamp passes through the blood vessel and impinges upon a photoelectric cell. The haemoglobin concentration of blood is so high that with a light path of from 1 to 2 mm. length, such as was used in these experiments, practically all light except the red is completely absorbed by both oxygenated and reduced blood. In other words, the blood acts itself as a red colour filter. Some experiments were made in 1936 by the "opened blood vessel" method, in which the animals were heparinized, and a small double-ended glass cannula was inserted in the carotid, the light passing through this glass tube instead of through the artery itself. This enabled us to use the large units (trough thicknesses of about 2 mm.) with which much experience had been attained in dog work, there being at that time no smaller units available. By 1937, however, smaller and lighter units suitable for arteries of 1·5–2 mm. in diameter (trough thicknesses 1·2–1·5 mm.) had been developed. Several advantages were obtained by using unopened blood vessels:

(1) The operation was rendered much simpler and quicker, which was of great importance for these experiments, where time is a vital factor.

(2) Heparin was unnecessary.

(3) It was found that there was less danger of kinking of the blood vessels, with subsequent blocking or settling of corpuscles, followed often by clotting even in the heparinized animal. These altered the optical conditions, making the method unreliable. Since the animals were in most cases under a local anaesthetic and not a general one, it was impossible to avoid a good deal of movement, and it was found absolutely essential to bind the element firmly in place in the neck (muscle and trachea sewed together with a unit in the middle).

Simultaneously with the oxygen record, a record of respiration was taken by means of a tambour connected to a cannula in the trachea. The tracing is really one of the alterations of intratracheal pressure; in as much as the nose may be more open in the later periods of the experiment than in the earlier ones, and on account of other variables, the tracing cannot be taken as a strict basis for any estimate of the volume of air which passes through the trachea.

Calibration. The instrument for recording oxygen saturation was calibrated by means of samples withdrawn from the other carotid, the content being determined either by the Van Slyke method or by a micro-modification of Kramer's photometric method (0·1 c.c. samples) or both. Whenever possible a first sample was drawn before the cord was tied, in order to make sure that at least one point on the calibration curve remained constant throughout the experiment, and an additional number of samples were drawn at the end of the experiment. In order to obtain a wide range of saturations for the calibration points, the animal was given oxygen or nitrogen to breathe. It was found that with oxygen breathing, the blood in the carotid always became practically fully oxygenated, so that simply by administering oxygen we were able to obtain the "100 % saturation" value at any time during the experiment.

The absolute accuracy in the experiments quoted in this paper was about 10 % saturation and in the bad ones about 20 %, though changes taking place over say a 5 min. period could be followed with an accuracy several times as great as this.

Sheep no. 215	Sample	Van Slyke value % sat.	Photocell method	Difference
	1	48	41	7
	2	57	46	11
	3	72	78	6
	4	49	42	7
	5	76	78	2

The above figures from samples taken at 15 min. intervals do not mean that there is a random error of anything up to 11 %, but that there is a drift in the error. Thus the first sample shows the photocell to give a reading 7 % lower than the Van Slyke, the second 11 % lower. But if a sample were taken immediately after the first it would have an error of only slightly over 7 %, and correspondingly a sample taken just before the second would be slightly below 11 %, so that two readings of the photocell taken within a short time, say 5 min., are closely comparable, though they may both be too low or too high.

EXPERIMENTAL RESULTS

The ultimate degree of oxygenation of the blood which reaches the brain is known approximately. The haemoglobin is in the region of 95 % saturated and the oxygen pressure is 70 mm. or over.

In the foetus of the sheep at or near term, results obtained in the laboratory indicate a good deal of variation. This is not unlikely, for the maternal circulation through the placenta may easily be affected by that variable quantity, the tension of the uterine wall. For example, in foetus 162 at 141 days the haemoglobin was 59 % saturated, and in foetus 74 at 144 days only 39 % saturated.

It must be stated quite clearly that these figures do not purport to indicate normal carotid saturations of the foetus when the sheep is in its normal condition: indeed we know that under conditions of intra-uterine operation the carotid blood between the 75th and 140th day is usually between 60 and 90 % saturated. They are those of foetuses during Caesarean section, in which the uterus therefore has been opened, and at a time when it is peculiarly sensitive to traumatic conditions. The point in quoting them is that during birth the oxygen saturation does fall as low as the figures quoted and from thence has to rise. It is that rise which is the subject of our investigation.

Four estimations of the percentage saturation at the commencement of experiments with the continuous registration method gave saturations as follows:

Foetus	215	198	199	193
Age in days	138	143	144	144
% saturation of carotid blood	35	48	32	70

Two experiments are illustrated in Figs. 1 and 2; they show the alterations in the percentage saturation of the carotid blood, consequent on tying the umbilical cord. They are on nos. 199 and 215 respectively. The two experiments differ in that no. 199 initially breathed air; it is selected, in fact, as being the experiment in which the oxygen saturation rose most rapidly when oxygen was inhaled. No. 215 was an experiment in which oxygen was inhaled from the moment of tying the cord, the lamb's head being thrust into a bag of oxygen. The respirations are also shown in these records. Fig. 3 shows the oxygen records transcribed and translated to a scale in which equal increments of percentage saturation are represented on a rectilinear scale. In Figs. 1 and 2 that is not so: the scale is more open at the high than at the low percentage saturations and is

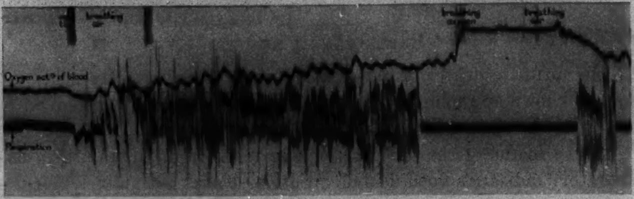

Fig. 1. Oxygen saturation of blood and respiration of lamb 199. The method of administering oxygen made it temporarily impossible to record the respiration.

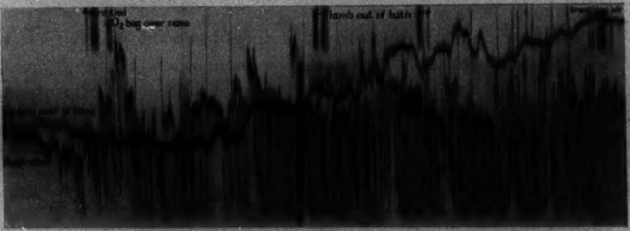

Fig. 2. Oxygen saturation of blood and respiration of lamb 215. Sensitivity of apparatus differed from that in Fig. 1.

therefore less easy to interpret. These records may be considered (1) in their more general aspect, and (2) in respect of certain interesting details which they represent.

(1) *In their general aspects* both curves indicate:

(*a*) About the same percentage saturation before the cord is tied, namely in the region of 35 % which is rather low.

(*b*) After the cord is tied there is a fall in each curve.

This fall is practically universal over all the curves which we have taken, though it varies in duration and in depth. The depth is largely a matter of the activity of the foetus and the duration of the dullness of its central nervous system. In the two experiments above, taking 35 %

saturation as being the initial value, 199 never fell below that value after the first 60 sec. and 215 never fell below it after 67 sec. from tying the cord. One may say that with an active foetus the saturation may fall

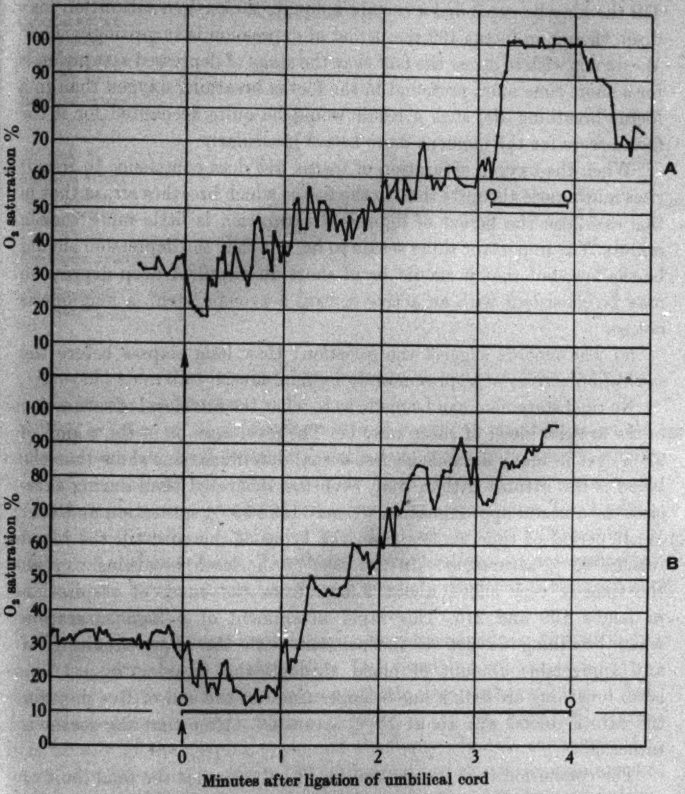

Fig. 3. Interpretation of oxygen saturation records in A, Fig. 1, lamb 199; B, Fig. 2, lamb 215. † Cord tied; 0——0, lamb breathing oxygen.

to a low level, but the fall is followed by a rapid rise and within about a minute and a half the saturation has reached a sustained value above that before the cord was tied.

The percentage saturation of the blood at any moment depends upon two main factors, the amount of oxygen which passes through the lung into the blood and the amount of oxygen used by the tissues. If the latter exceeds the former the saturation of the arterial blood must drop. Till the lung be expanded a certain amount, the oxygen saturation may drop, though in foetus 199 the period of depression is surprisingly short. We do not wish to stress the fact that the stage of depressed saturation is for a short time more profound in the foetus breathing oxygen than in a foetus breathing air; such a result would be quite accounted for if the foetus were for the moment more active muscularly.

When the oxygen saturation of foetus 215 does commence to rise, it rises much more abruptly than in the foetus which breathes air, so that in this case also the period of depressed saturation is little more than a minute. The important thing seems to be, not that the depression should be shallow, but that it should be of short duration. A deep depression may be consistent with an active central nervous system, a long one is not so.

(c) The records suggest the question: How long elapses before the saturation arrives at approximately its final level?

No rigid statement can be made as to what the final level of saturation in the arterial blood of sheep may be. The level may be in the region of 97 %, yet in many animals at rest actual determinations show that the blood is less saturated than that, even less saturated than during exercise. As a useful approximation we may take 95 % saturation and ask: What period of time elapses from the tying of the cord till the blood reaches 95 % saturation? In the case of the lamb breathing oxygen the time is very short, about 5 min. from the onset of respiration in lambs 199 and 215. This rapid attainment of a high saturation when breathing oxygen is inconsistent with the short-circuiting of any appreciable amount of blood through the foramen ovale. The lamb breathing air took a much longer time: at the end of five minutes the carotid blood was about 75 % saturated. After that the ascent is rather slow.

The conclusion that the arterial blood only reaches the final limit of saturation rather slowly is borne out by results of arterial puncture as is shown in sheep 92. The foetus was born by Caesarean section at 144 days, under urethane. In appraising the results tabulated below (Table I) it should be remembered that in the sheep the brachials and the carotids of both sides come from common trunks, while, of course, the umbilical and femorals come from the dorsal aorta.

TABLE I. Oxygen in arterial blood of lamb breathing air (content and capacity expressed in c.c./100 c.c. of blood)

Time after tying cord ...	½ hr.	1 hr.	5 hr.	48 hr.	6 days
Carotid artery:		Head and arms			
Content	—	—	—	—	11·9
Capacity	—	—	—	—	13·3
Sat. %	—	—	—	—	90
Brachial artery:					
Content	—	14·1	15·0	13·1	—
Capacity	—	17·1	15·8	14·8	—
Sat. %	—	83	96	92	—
		Body			
Femoral artery:					
Content	—	—	16·3	14·6	12·2
Capacity	—	—	16·4	14·9	13·3
Sat. %	—	—	99	98	92
Umbilical artery:					
Content	15·6	—	—	—	—
Capacity	18·3	—	—	—	—
Sat. %	85	—	—	—	—

It would seem that in somewhere between an hour and 5 hr. the blood reaches its final saturation.

(2) *The effect of single respirations and groups of respirations on the oxygen saturation.* Exp. 199 shows particularly well a phenomenon which is of fairly constant occurrence, namely more or less rhythmic waves in the saturation curve associated with groups of respirations. The typical group of respirations consists of one deep respiration followed by a number of shallower ones. The shallower respirations do not suffice to maintain the oxygen saturation, which tends to drop but is restored by the deep respiration.

We have here two different processes, the occasional gasp thrown in on top of the normal type of respiration. It is possible to obtain either type separately. If, instead of a local anaesthetic, the mother is given a full dose of urethane (1 g./kg.), when respiration in the new born lamb starts it is usually of the gasping type, and that only. In this connexion the onset of respiration in sheep 189 is instructive: the sheep was anaesthetized as described; 40 sec. elapsed from the tying of the cord before the foetus breathed; it then gave a gasp followed at intervals by other gasps. It was not for three minutes that the first sign of respiration of the normal type appeared; a few shallow breaths were given, but the phenomenon disappeared: at intervals the normal type of respiration reappeard again to fade and leave the animal dependent upon its gasps.

That this is the typical onset of respiration under a full dose of urethane is sufficiently attested by the fact that in earlier experiments on goats, in which that anaesthetic was used throughout, one of us [Barcroft] was misled into supposing it to be the normal onset and, as the gasps seemed to be caused by lack of oxygen, he supposed that want of oxygen was the normal stimulus to the establishment of respiration.

That the gasps are due in some way to want of oxygen is shown by the fact that they can be induced by agents which produce methaemoglobin in the blood and abolished by the subsequent injection of a copious supply of red corpuscles [Barcroft & Barron, 1935].

SUMMARY

1. Continuous tracings of the oxygen saturation in the carotid blood have been taken during the birth of lambs by Caesarean section.

2. The oxygen saturation, probably already unduly low owing to the traumatic condition of the uterus, falls after the cord is tied to perhaps between 10 and 20 % saturation. This phase in favourable conditions lasts about a minute, though the period varies considerably.

3. In lambs which breathe air the saturation may rise to about 75 % in five minutes from tying the umbilical cord, but probably does not reach 95 % saturation for about 5 hr.

4. The administration of oxygen after 4 min. and when the saturation was 60–70 % at once raised it to about 100 %, showing that there is no great amount of circulation through the parts of the lung not accessible to gas. It does not prove, however, that the whole lung has expanded.

5. When oxygen is administered from the start, 95 % saturation may be realized within 5 min. This is inconsistent with the short-circuiting of any amount of blood through the foramen ovale so great as to be physiologically important [see Barclay & Franklin, 1938].

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Afferent fibres of the hypoglossal nerve. By C. B. B. Downman.

(*From the Sherrington School of Physiology, St Thomas's Hospital*)

Investigations were carried out to demonstrate the existence of afferent fibres in the hypoglossal nerve. Reflex rises of blood pressure in the chloralosed or decerebrated cat, and dilatation of the pupil in the chloralosed cat [Irving & McSwiney, 1935] were used as indices of afferent fibres. The arrangement of the XIIth nerve in the cat is in general similar to that described for man.

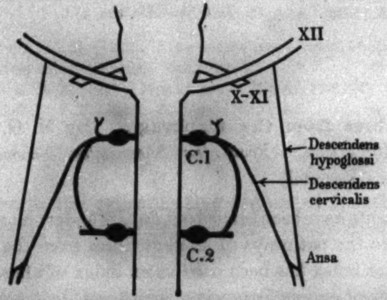

Fig. 1. General plan of the hypoglossal nerve of the cat. Note origin of descendens cervicalis from anterior ramus of C. 1; no direct connexion between C. 1 and the stem of XII could be found.

Stimulation (mechanical or electrical) of the central cut end of the hypoglossal nerve close to the tongue caused a rise of blood pressure and dilatation of the pupil. From the following evidence it is concluded that the afferent fibres involved pass directly into the medulla: (*a*) after section of the spinal cord at C. 2 and removal of the 1st and 2nd cervical dorsal root ganglia, stimulation of the central end of the hypoglossal nerve caused dilatation of the pupil, (*b*) the responses were abolished by section of both the Xth and XIIth nerves. In some animals the afferent fibres entered the medulla via the Xth nerves; while in others it was possible to demonstrate that some fibres at least entered with the XIIth nerves.

It was also demonstrated that afferent fibres are present in the descendens cervicalis, and that these fibres enter the spinal cord by the 1st cervical roots. Afferent fibres were also identified in the descendens hypoglossi.

These findings demonstrate clearly the existence of afferent fibres in the hypoglossal nerve of the cat, thereby confirming and extending the work of Tarkhan [1936] and Langworthy [1924]. Attempts were made to stimulate the end-organs of these afferent fibres, but without success. After section of the chorda-lingual and glosso-pharyngeal nerves, crushing, cutting, burning, stretching of the tongue produced no reflex response. It is therefore impossible to assign any specific role to the afferent fibres.

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Afferent fibres from the diaphragm.

By M. G. A. Little and B. A. McSwiney. (*From the Sherrington School of Physiology, St Thomas's Hospital*)

Experiments have been carried out on cats under chloralose anaesthesia to investigate the pathways of afferent fibres from the diaphragm. The pupillo-dilator reflex has been used as an index of afferent impulses.

Two series of experiments were carried out:

(*a*) To demonstrate the presence and course of afferent fibres in the phrenic nerves, and

(*b*) To demonstrate the afferent innervation of the diaphragm.

In the first series the phrenic nerves were ligatured and cut in the thorax and stimulated by faradic shocks or by pinching. Pupil dilatation was thus elicited, and by similar operations to those described in previous publications [McSwiney & Suffolk, 1938] information was obtained regarding the entry into the spinal cord of the afferent fibres subserving this reflex. The afferent fibres in the phrenic nerves were found to enter the spinal cord via the dorsal roots of the fifth and sixth cervical segments.

In the second series of experiments the reflex was obtained by stimulating either the pleural or the peritoneal surfaces of the diaphragm by different methods.

Application of acid or alkali, or of thermal stimuli, produced pupil dilatation. Pinching of the diaphragm also elicited the response. Stimulation of the diaphragm with a unipolar electrode was found, however, to be the most efficient method.

Evidence was obtained that afferent fibres of the pupillo-dilator reflex, innervating the diaphragm, were present in the phrenic and intercostal nerves. The afferent fibres in the phrenic nerves form the main pathway for afferent impulses from the diaphragm.

No segmental innervation of the different quadrants of the diaphragm was found and no great overlap in the afferent innervation of the two halves of the diaphragm could be observed.

Hinsey & Phillips [1937] and Capps [1932] amongst others have shown that the phrenic nerves may convey noci-ceptive impulses from the diaphragm.

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Water-retention in the reproductive organs of female monkeys.

By S. ZUCKERMAN. (*From the Department of Human Anatomy, University of Oxford*)

Like the hormone of the adrenal cortex, oestrogenic hormone [Krohn & Zuckerman, 1937; Thorn & Harrop, 1937; Guthkelch & Zuckerman, 1937], testosterone and progesterone [Thorn & Harrop, 1937] have water- and salt-retaining properties. The fact that swelling of the sexual skin does not normally occur during the post-ovulation phase of the menstrual cycle in the pig-tailed macaque shows that the progestin produced by the corpus luteum is unable to retain in the sexual skin the water deposited there as a result of oestrogenic stimulation in the pre-ovulation phase. A series of experiments carried out on spayed pig-tailed macaques which had first been injected with oestrone in order to induce sexual-skin swelling shows that neither testosterone propionate (100 mg. every third day) nor cortin (10 c.c. daily of "Eucortone", Allen and Hanbury, or "Cortin", Upjohn Company) is able to prevent the normal excretion of sexual-skin water once the oestrogenic stimulation has ceased. On the other hand, the injection of pitressin at short intervals does delay the subsidence of the swelling.

Uterine bleeding in monkeys is associated with dehydration of the uterus [van Dyke & Ch'en, 1936]. A series of experiments on spayed pig-tailed and rhesus macaques shows that unlike progesterone or testosterone propionate, neither cortin nor pitressin is able to inhibit post-oestrin bleeding.

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The role of carotid sinus and vagal chemoreceptors in the respiratory and vasomotor effects of hypoxaemia in anaesthetized and normal dogs.

By J. J. BOUCKAERT, C. HEYMANS and ADLI SAMAAN. (*From the Department of Pharmacology, University of Ghent, Belgium*)

It has been shown that, in anaesthetized animals, the respiratory and vasomotor stimulation induced by hypoxaemia is not of direct central origin, but is mainly due to reflex excitation of the centres through the cardio-aortic and carotid sinus chemoreceptors [J. F. & C. Heymans, 1927; C. Heymans, Bouckaert & Dautrebande, 1930; Owen & Gesell, 1931; C. Heymans, Bouckaert, v. Euler & Dautrebande, 1932; Selladurai & Wright, 1932; v. Euler & Liljestrand, 1936; Jongbloed, 1936; Comroe & Schmidt, 1938; Bernthal, 1938; Mertens, 1938; Lambert & Gellhorn, 1938].

Decharneux [1934] and Dautrebande [1937] asserted, however, that hypoxaemia stimulates also directly the respiratory centre of the unanaesthetized dog deprived of its chemoreceptors.

Several series of experiments with different techniques were performed in order to examine this assertion.

In these experiments we observed that, in the unanaesthetized or decerebrate dog, as in the anaesthetized (chloralosane) animal, the inhalation of a gas mixture (7–12% O_2 in N_2) provokes a marked increase in the total pulmonary ventilation and a rise in general blood pressure, when the carotid sinus nerves only, or the vagi only, or both are intact. When these nerves are cut, hypoxaemia induces, on the contrary, a reduction in the total ventilation and a fall in blood pressure. Inhalation of 7–10% oxygen may provoke rapidly a deep depression and even a paralysis of the respiration and circulation. In the unanaesthetized dog, however, this primary depression of respiration and blood pressure may be followed or interrupted by a secondary increase of total ventilation and a rise of blood pressure. These secondary stimulations of the respiratory and vasomotor centres by hypoxaemia in the non-anaesthetized animal are related to psycho-motor reactions as a consequence of the primary inhibition. These secondary reactions are depressed or suppressed by narcotics, anaesthetics or decerebration.

These experiments thus show that in the non-anaesthetized dog as well as in the anaesthetized animal, hypoxaemia stimulates the respiratory and vasomotor centres primarily by means of the carotid sinus and vagal nerves, the chemoreceptors of which, as shown previously, are located in the carotid body and cardio-aortic vascular area. The role of the vagal chemoreceptors is not as important as that of the carotid sinus. In anaesthetized or decerebrate animals deprived of these chemoreceptors, hypoxaemia depresses the respiratory and vasomotor centres; in non-anaesthetized animals, this primary central depression may be followed by a secondary, indirect, stimulation.

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Anti-cholinesterases and the rate of decay of the synaptic transmitter in the superior cervical ganglion. By J. C. Eccles. (*From the Kanematsu Memorial Institute of Pathology, Sydney Hospital, Sydney*)

Recently Rosenblueth & Simeone [1938] claim to have shown that in the superior cervical ganglion the anti-cholinesterases, eserine and prostigmin, slow the rate of decay of the synaptic transmitter, thereby supporting the hypothesis that this transmitter is acetylcholine. Their experiments are in some measure a repetition of experiments [Eccles, 1937] in which an unsuccessful attempt was made to detect such an action of eserine, and they attribute their success to the massive doses of anti-cholinesterases which they employed.

However, their experiments also differed in that, when attempting to measure the rate of decay of the transmitter, they investigated the interaction on the ganglion cells of two preganglionic volleys set up by *maximal* stimulation of each branch of the annulus Vieussens. On the other hand, Eccles had purposely used weak submaximal stimuli, for with maximal stimulation the large overlap in the fields of ganglion cells excited to discharge from each branch would introduce a complicating response [Eccles, 1935] preventing any determination of the rate of decay of the transmitter. Thus, when the conditioning and testing volleys are maximal and almost simultaneous, subtraction of the conditioning response from the combined response, monophasically recorded, gives a wave having a crest followed by a trough (cf. Text-fig. 11 [Eccles, 1935]). Its diphasic character indicates that this wave is not due to the response of additional ganglion cells (spatial facilitation), but must arise on account of some ganglion cells discharging impulses earlier with the combined than with the conditioning response, the forward displacement of their response giving in the subtracted curve the crest with the later trough. The synaptic delay of the response to the conditioning volley must be shortened by the almost simultaneous arrival of impulses of the testing volley. Lengthening of the interval between the conditioning and testing volleys causes this diphasic wave rapidly to diminish and it disappears at about 2 msec. interval, the impulses of the testing volley then arriving too late to affect the responses of the ganglion cells to the conditioning volley. Obviously this decline bears no relationship whatever to the decline of the spatially *facilitated* responses which occurs when the interval between two *submaximal* volleys is progressively increased, and which provides a measure of the rate of decay of the detonator response of the ganglion cells, and hence indirectly of the synaptic transmitter [Eccles, 1937].

Thus the period of decline in Rosenblueth & Simeone's curves has no relationship to the decay of the synaptic transmitter; hence the slowing of the decline sometimes produced by anti-cholinesterases does not indicate a slowing in the rate of this decay, i.e. their experiments have no significance as a test for any action which anti-cholinesterases may have on the synaptic transmitter. Possibly the slowing in decline which they observed may have been caused by the lengthening of synaptic delay which seemed to result from their massive doses of eserine or prostigmin (cf. their Fig. 1). However that may be, it must be concluded that no evidence has yet been adduced to show that anti-cholinesterases have any action in slowing the rate of decay of the synaptic transmitter in the superior cervical ganglion, and hence the hypothesis that acetylcholine is the synaptic transmitter still lacks this fundamental supporting evidence.

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Responses evoked by a nerve volley in mammalian striated muscle. By J. C. Eccles and W. J. O'Connor. (*From the Kanematsu Memorial Institute of Pathology, Sydney Hospital, Sydney*)

Responses of cat's tibialis anticus (*T.A.*) and soleus (*S.*) muscles have been evoked by one or two maximal motor nerve volleys and recorded optically by mirror myograph and cathode-ray oscillograph. In some experiments by cutting down the motor nerve the response has been restricted to a small superficial bundle of muscle fibres having a sharply localized group of motor end-plates.

A nerve volley often sets up the discharge of a single impulse along every muscle fibre, the extreme ranges of the neuro-muscular delays being 0·7–1·5 msec. (*T.A.*) and 0·8–1·6 msec. (*S.*). These impulses are propagated along the muscle fibres at about 6 (*T.A.*) and 4 (*S.*) metres a second. After this response a second (testing) nerve volley finds the muscle unresponsive for about 2·0–2·5 msec. (*T.A.*, *S.*), and then relatively unresponsive, but there is a later period, up to 8 msec., during which a slight shortening (0·1–0·2 msec.) of the neuro-muscular delays (particularly the longer) indicates a supernormal excitability of the end-plates. Finally there is a still further period, up to at least 40 msec., during which a second muscle impulse exhibits a supernormal conduction velocity. When the testing nerve volley reaches the motor end-plates during the latter part of the muscle's unresponsive period, it may still evoke the discharge of muscle impulses at the end of this unresponsive period by virtue of a delayed excitatory effect, the maximum lengthening of the normal neuro-muscular delay being about 1·1 msec. (*T.A.*) or 0·8 msec. (*S.*), values indicative of the rapid dissipation of the excitatory action which a nerve impulse exerts on a motor end-plate.

Fig. 1. *T.A.* Repetitive action potential to a single nerve volley.

Fig. 2. *T.A.* Summation of two volleys at various intervals to produce repetitive response. Time = 10 msec.

In many experiments with a single motor nerve volley the initial discharge of muscle impulses is followed by a repetitive discharge from a few motor end-plates, and in some experiments almost all end-plates may respond in this way (Fig. 1). These repetitive responses do not arise in the nerve and great care has been taken to avoid muscle injury. With *T.A.* the repetitive discharge may persist for as long as 120 msec., the initial frequency of about 200 a second falling to as low as 100. On the other hand, with soleus the frequency is as high as 300 a second, but the discharge ceases within 20 msec. With two nerve volleys at less than 8 msec. apart, this repetitive response is exhibited by many additional muscle fibres, the maximal summation being observed at volley intervals from about 1·0 to 3·0 msec. (Fig. 2). With longer volley intervals (8 msec. to as much as 500 msec.) the second volley evokes a diminished repetitive response. Thus in every respect these repetitive responses of normal muscles closely resemble the repetitive responses which nerve impulses set up in eserinized muscles [Brown, Dale & Feldberg, 1936; Brown, 1937].

During the repetitive response there is a tendency for the individual muscle fibres to beat in phase (Fig. 1). Since this synchronization returns after temporary interference, there must be a process whereby impulses in some muscle fibres condition responses in other fibres; and it has been possible to demonstrate that this process actually is responsible for evoking repetitive responses in many muscle fibres which otherwise would give only the initial response. Since this excitatory action of some muscle impulses on the end-plates of other muscle fibres must be exerted within about 1 msec., direct electrical spread of the muscle action currents provides the only probable basis for such a rapid spread of excitatory action. On such an explanation the end-plates must be very easily excited electrically.

With two nerve volleys at a very short interval (0·5–2·0 msec., *S.*) a very brief depression of repetitive response follows the second volley but immediately precedes the summed repetitive response.

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Action potentials evoked by indirect stimulation of curarized muscle. By J. C. Eccles and W. J. O'Connor. (*From the Kanematsu Memorial Institute of Pathology, Sydney Hospital, Sydney*)

Soleus muscles were cut down as in the previous communication and by moving the leading electrodes the site of origin of the muscle impulses, i.e. the motor end-plates, could be determined, and in our best experiments was found to be localized to an area of only a few millimetres in diameter.

When a dose of curare had been given so that nerve stimulation failed to produce any contraction, it still evoked two kinds of action potentials in the muscle in addition to the potentials of the motor nerve impulses. Both these potentials are confined to the region of the end-plates, which becomes negative relative to the rest of the muscle, and both have the same latent period as the normal muscle spike, but they are easily distinguishable by varying the depth of curarization.

(1) With deep curarization the slow potential (which may be as large as 10% of the normal muscle spike potential) is alone detectable (Fig. 1), its size being diminished, but its time course unaltered, by further curarization. A second nerve volley at intervals longer than about 0·8 msec. and even beyond 40 msec. produces an additional wave of

similar shape but smaller (about 80%) potential. This slow wave appears to be associated with three excitatory effects which nerve impulses evoke at the region of the neuro-muscular junction. (*a*) The slight shortening of neuro-muscular delay (see previous communication). (*b*) The repetitive response which one or two nerve volleys set up sometimes in normal muscle (see previous communication), and invariably in muscles adequately treated with anti-cholinesterases. (*c*) In a curarized muscle the removal of neuro-muscular block, which is revealed by a testing volley at short intervals (0·8–8 msec.) after a conditioning volley (cf. Bremer's *addition latente*).

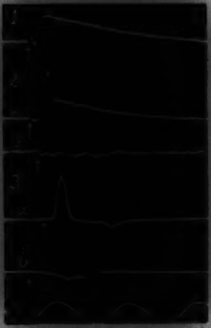

Fig. 1. Full curarization showing the slow wave alone. Amplification, 50 times Fig. 3.

Fig. 2. (*a*) and (*b*). Less curarization showing (*a*) the spike response at the end-plates; (*b*) no potential at 2 cm. from the end-plates. No contraction. Amplification, (*a*) 25; (*b*) 75 times Fig. 3.

Fig. 3. (*a*) and (*b*). Corresponding records before curarization. Time = 10 msec.

(2) With lighter curarization a brief sharp spike is superimposed on the early part of the slow potential (Fig. 2). Its shape and duration are identical with the spike produced by propagated muscle impulses in normal muscle (Fig. 3), and recording with leads away from the end-plate region shows an increased delay indicating propagation, but this spike potential is propagated no more than 5 mm. (probably much less) along the muscle fibres before dying out. Also in some experiments it has been possible to demonstrate by a testing volley that an unresponsive period of about 2·5 msec. follows the spike potential; however, most experiments are confused by the summation associated with the above slow wave, the testing volley then setting up spike potentials in fresh muscle fibres.

These spike potentials would thus appear to be due to impulses which arise in the curarized muscle fibres in the region of the end-plates, but which die out after propagating at most a few millimetres. As recovery from curarization proceeds, fully propagated impulses reappear, apparently being recruited from the abortive group. The contraction of the muscle is proportional to these fully propagated impulses, the abortive impulses giving no detectable contraction. Abortive impulses may produce a spike potential as high as 30% of the maximum spike of normal muscles.

Rushton [1937] has recently shown that, on the electrical theory of the nerve impulse, a length of excited nerve fibre less than a critical value would result in a decremental propagation of an impulse, terminating in its rapid extinction; and Hodgkin [1937] has recorded the monophasic spike potentials produced by such decrementing impulses. The abortive muscle impulses arising from the end-plates of curarized muscle would seem to be another example of this phenomenon, the stimulating action of the nerve impulse at the motor end-plate being inadequate to excite a length of the muscle fibre sufficiently large to establish an all-or-nothing impulse. The absence of abortive impulses with deeper curarization of the muscle corresponds to the failure of a stimulus of less than half-threshold to set up abortive impulses in nerve [Katz, 1937; Hodgkin, 1937].

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"Free" and "bound" histamine and acetylcholine. By E. R. Trethewie. (*From the Walter and Eliza Hall Institute, Melbourne*)

The presence in the cell of two forms of acetylcholine, "free" and "bound", has been claimed by Corteggiani [1937], by Corteggiani, Gautrelet, Kaswin & Mentzer [1936] and by Corteggiani, Gautrelet, Gautel & Kaswin [1937]. They assume that part of the tissue acetylcholine is bound in a "complex", which may be destroyed or from which the acetylcholine may be split off under certain conditions. Their conclusions are based upon the findings that the acetylcholine yield of an eserinized saline extract of ground-up tissue increases on heating and on standing. We have confirmed their results for the guinea-pig's brain and have also shown that the histamine yield of saline extracts of ground dog's liver increases under similar conditions. The increase, however, does not result from the presence of a soluble "complex" from which acetylcholine or histamine is given off, but from the fact that in the extracts there is suspended incompletely fragmented cellular material which retains its histamine and acetylcholine. The acetylcholine and histamine yield of cell-free extracts obtained by centrifugation did not increase on heating or standing whereas the spun-down particles yielded histamine or acetylcholine on heating, and the amounts so obtained corresponded to the increased yield when the non-centrifuged extract was heated.

For instance, a guinea-pig's brain, exclusive of the cerebellum, was ground up with quartz powder in eserinized Tyrode solution and divided into two equal parts. One was assayed without being centrifuged; it yielded 1·2μg. of acetylcholine per g., this value rose to 5·6μg. when the extract was heated to 70° C. for 10 min. The other portion was centrifuged and the unheated and heated supernatant fluid, corresponding to 1 g. tissue, revealed an acetylcholine equivalent of 1·5 and 1·4μg. respectively. Sediment, corresponding to 1 g. of tissue, yielded 3·9μg. acetylcholine on heating. Similarly a piece of dog's liver was ground up in Tyrode solution with quartz powder and divided into two equal parts. On heating the non-centrifuged extract the histamine equivalent rose from 14 to 64μg./g.; the histamine equivalent of the supernatant fluid of the centrifuged part was 11μg., and when sediment corresponding to 1g. tissue was taken up in Tyrode solution and boiled, 53μg. of histamine were brought into solution. Microscopical examination of unheated non-centrifuged extracts of dog's liver revealed the presence in the solution of cell conglomerates, individual cells, and cell debris. Incomplete spinning down of the cellular material left the debris suspended in solution. Such debris is rich in histamine which is brought into solution on heating.

Our results show that the cell cannot be regarded as a "diffusion-tight compartment" in the interior of which histamine or acetylcholine is freely diffusible. There is no experimental evidence for the assumption of a "precursor" or "complex". The internal structure of the cell and not the integrity of the outer cell membrane determines its capacity for holding in its substance these active principles. As long as the internal structure even of part of a cell is intact, acetylcholine or histamine is prevented from diffusing readily.

Our results may have some bearing on recent observations on formation of acetylcholine. Those of Chang, on the placenta [1935], and of Stedman and Stedman, on the brain [1937], were carried out with saline extracts of minced tissue and the possibility that their "formed" acetylcholine is derived from suspended cellular material has not been excluded. Stedman and Stedman state that "mincing the brain tissue causes a complete destruction of any acetylcholine which it may have contained". This we have shown is not true. Since suspended cell debris gives off its acetylcholine on standing the thin slices of brain used in the experiments of Quastel, Tennenbaum & Wheatley [1936] might have behaved similarly.

A full account of the experiments will appear in the *Australian Journal of Experimental Biology and Medical Science*, vol. 16.

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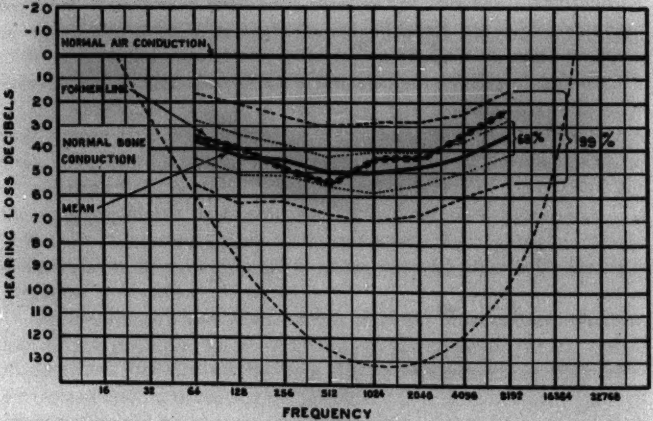
Normal hearing by bone conduction. By A. Greenbaum, P. M. T. Kerridge and E. J. Ross. (*From the Department of Physiology, Pharmacology and Biochemistry, University College, London*)

Deviations from normal hearing by bone conduction are used in otology as indications of defects in the cochlea or in the auditory nervous pathway. It is therefore important to know the range of normal hearing by bone conduction, as well as the mean value. Such information has not previously been available.



Determinations of bone conduction hearing were made on 100 students of both sexes, between 18 and 25 years of age, who had no history of ear disease. The measurements were made in the sound-testing room of the Royal Ear Hospital, with two pure tone audiometers made by the Western Electric Company, models 2B and 2BE respectively.

The results are summarized in the accompanying figure, which also shows the line given by the above firm as representing normal bone conduction. This "former line" was found by testing the hearing of 30 young American adults in a sound-proof room.

The action of cell-free muscle extract on insulin. By H. LEHMANN and H. SCHLOSSMANN. (*From the Biochemical and Physiological Laboratories, Cambridge*)

In cell-free extracts of rabbit's muscle we have found two principles which inactivate insulin. The one passes through a semipermeable membrane and is thermostable, whereas the other does not dialyse and is destroyed at or above 80°.

The first principle is probably glutathione or cysteine. These sulphydryl compounds occur in muscle. They are known to inactivate insulin by reducing its —SS— groups, themselves being oxidized, according to Hopkins' equilibrium [Vignaud, Fitch, Pekarek & Wayne-Lockwood, 1931].

When the first principle is removed by dialysis and the extract heated for a few minutes, the injection of this preparation + insulin, after incubation for 30 min. at 37°, has the same effect on the rabbit's blood sugar level as insulin alone. But when insulin is added to the dialysed extract, incubated for 10–30 min., and then heated, it can be seen from the effect on the blood sugar level that most of the insulin has been inactivated by the second principle.

The nitroprusside colour reaction demonstrates that —SS— linkages in the insulin molecule can also be reduced to —SH groups by the second principle. The fact that animal tissues destroy insulin has hitherto been attributed to their proteolytic power [Schmidt & Saatchian, 1929].

Both insulin hydrochloride and zinc-insulinate (crystalline insulin) are inactivated by the thermolabile principle, but there is a difference in the rate of destruction, the latter being much less inactivated than the former.

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Carbonic anhydrase in the gastro-intestinal mucosa. By H. W. Davenport and R. B. Fisher. (*From the Department of Biochemistry, Oxford*)

In an attempt to investigate the mechanism of HCl production in the stomach we were led to look for carbonic anhydrase in the mucosa of the gastro-intestinal tract. As circumstances prevent the continuation of this work in collaboration we wish to record here our preliminary observations.

Extracts of mucosa were prepared in the following manner. After the animal had been killed by beheading or a blow on the back of the neck the abdominal viscera were freed from blood by perfusion with 0·9% NaCl, the contents of the stomach and intestine were washed out, and the mucosa was carefully scraped off and weighed. The tissue was then ground with sand and suspended in 4 vols. water. 1 vol. of alcohol and 1 vol. of chloroform were added and the whole shaken thoroughly and centrifuged for 10 min. The carbonic anhydrase activity of the aqueous layer was determined by the method of Philpot & Philpot [1936]. Dr F. J. W. Roughton kindly enabled one of us (H.W.D.) to determine the activity of a dry preparation of cat gastric mucosa by the manometric method of Meldrum & Roughton [1933], and we have used the data so obtained to convert the activities of our preparations into units comparable with those of Meldrum & Roughton. Typical data are given in the table:

	Rat	Cat	Rabbit
Stomach:			
Cardiac third	2·5	5·8	1·5
Middle third	2·5	4·9	1·7
Pyloric third	2·3	3·4	2·0
Duodenum	0·8	0·6	0·5
Ileum	? +	0·8	0·5
Pancreas	+	0·4	–

(The figures represent Meldrum & Roughton units per mg. wet weight)

Since these data are in terms of wet weight of oedematous tissue they compare remarkably with those of Meldrum & Roughton (*loc. cit.*) for the carbonic anhydrase activity of blood. These workers give their results in units per c.mm. of blood, and it is permissible to suppose that the activity in units per mg. of corpuscles will be approximately twice their published figures. As they find mean values of 1·2 units/c.mm. in rabbit's blood and 1·7 units/c.mm. in rat's blood, it is clear that the activity of the gastric mucosa is of the same order as that of the red cells in the species in which data for comparison are available.

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Inactivation of cobra venom by adsorption. By A. C. FRAZER and H. C. STEWART. (*From the Physiology Department, St Mary's Hospital Medical School, W.* 2)

Bacterial toxins can be rendered innocuous by addition of finely dispersed oil-in-water emulsions [Frazer & Walsh, 1934; Myers, 1934; Oerskov & Schmidt, 1935]. The detoxication is thought to be due to adsorption of the toxin at the oil/water interface [Frazer, 1936]. In this communication further evidence is put forward that the detoxicating mechanism is adsorption.

The activity of cobra venom is usually investigated by intraperitoneal injection in mice. In the first investigation of venom-emulsion mixtures made by this method all the animals died. This indicated that no protection was afforded by the emulsion. Two differences were noted between the venom-emulsion (V.-E.) animals and the controls. They were:

(1) Whereas the controls went immediately downhill and died in about 2 hr., the animals having the V.-E. mixture remained normal for an hour or more after injection and then rapidly succumbed.

(2) Post-mortem the abdominal cavities of the V.-E. group contained flaky white pieces which were not found in the controls.

The results indicated that the venom was actually inactivated by the emulsion but that it was subsequently liberated by the "breaking" of the emulsion.

A. *More accurate demonstration of the difference in the symptom-development-rate when venom or venom-emulsion mixture is given.* The curare-like action of cobra venom [Kellaway, 1937] results in the paralysis of the respiratory muscles, and the respiration rate of the mouse indicates the degree of intoxication, this always receives support from the general appearance of the mouse, its response to food and the approach of the observer. As the venom takes effect the respiration rate slows from the normal value of 200 per minute until gasping and intense dyspnoea with convulsions develop just before death. One group of mice is given mixtures of venom with alkaline soap solution identical with the continuous phase of the emulsion, and the second group receives mixtures of venom and emulsion. All the mixtures are incubated at 37° C. for 15 min. before intraperitoneal injection. The respiration rate is observed at regular intervals by accurately timing twenty respirations. The respiratory effects appear later but progress more rapidly in the animals receiving the V.-E. mixture.

B. *Why should the emulsion break after intraperitoneal injection?* The emulsions are prepared on a high-pressure emulsifying machine [Frazer & Walsh, 1933]. They are extremely stable, but creaming occurs with acidification or the addition of salt, due to interference with the soap stabilizer. Addition of normal saline diminishes the stability. After intraperitoneal injection the water phase is rapidly absorbed and the dispersed phase is concentrated. Transudation of fluid into the peritoneal cavity causes the final creaming of the emulsion and precipitation of the dispersed phase in white flakes. This creaming progressively decreases the size of the oil/water interface.

C. *Demonstration that the venom-emulsion mixture is non-toxic.* If two groups of rabbits are taken, A and B:

Group A receive intravenously a suitable dose of a mixture of venom with alkaline soap solution as in the previous mice experiments.

Group B receive intravenously a mixture similar to Group A except that emulsion is used in which the continuous phase is identical with the alkaline soap solution. The only difference therefore is that the B mixtures contain olive oil in an extremely finely dispersed state.

All the venom mixtures are incubated for 15 min. prior to injection.

Results: All animals of group A die within the hour. None of group B shows any ill effects. Many have had repeated doses and no toxic effects have been seen.

To summarize:

i. V.-E. mixtures are non-toxic; detoxication depends upon the presence of the finely dispersed oil globules.

ii. If the non-toxic V.-E. mixture is given intraperitoneally, the animal shows no symptoms for an hour or so, then they suddenly appear and the animal rapidly dies.

iii. Intraperitoneal injection of emulsion causes creaming and progressive diminution of the oil/water interface.

We conclude from these observations that the inactivation of venom by emulsion is due to adsorption at the oil/water interface, which is in accordance with experiments previously reported on the mechanism of detoxication of bacterial toxins.

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The inactivation of adrenaline by catechol oxidase *in vitro*.

By H. Blaschko and H. Schlossmann. (*From the Physiological Laboratory, Cambridge*)

Solutions of adrenaline in phosphate buffer of *p*H 7·4 were incubated with highly purified preparations of catechol oxidase from *Psalliota* and *Lactarius* (kindly given to us by Dr T. Mann). The uptake of oxygen was measured manometrically and compared with the degree of inactivation as determined on the arterial blood pressure of the decerebrate cat. Samples of the mixture were taken at different stages of the reaction.

The uptake of oxygen proceeds rapidly up to two atoms per molecule of adrenaline, resulting in the formation of adrenochrome, the red oxidation product of adrenaline [Green & Richter, 1937]. This is followed by a very much slower uptake of oxygen which is associated with a colour change from red to brown and brings the total oxygen uptake to 5–6 atoms.

The assay on the cats' blood pressure showed that the complete inactivation of adrenaline coincided with the termination of the phase of rapid oxygen uptake; the adrenaline activity fell off correspondingly with oxygen uptake, e.g. after the uptake of one atom of oxygen the remaining activity was about one-half of the initial activity of the sample. Samples were also taken during the slow phase of oxygen uptake and, when tested on the cats' blood pressure, at no stage showed either pressor or depressor action. Therefore the adrenoxine-effect recently described by Bacq [1938] cannot be due to the action of pure catechol oxidase on adrenaline. The slow oxygen uptake at *p*H 7·4 is non-enzymic and due to autoxidation of adrenochrome [Green & Richter, 1937].

This work has been carried out with a grant from the Ella Sachs-Plotz Foundation.

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The measurement of nerve impulse velocity. By Otto H. Schmitt.

(From the Department of Physiology, University College, London)

It can be shown [Hill, 1934] that the potential difference developed between two electrodes placed close together on an active nerve is proportional to the first derivative with respect to time of the monophasic action potential. Since the peak of the action potential is the point at which its slope changes sign, it follows that at this time the first derivative must cross the base-line. This intersection furnishes a very accurate index of the time of passage of the impulse peak past the electrode pair.

Fig. 1*a*.

Fig. 1*b*.

If two such pairs of electrodes are placed on a nerve and are led to a common amplifier through a suitable resistance network to prevent interactions between the two pairs, one has a very suitable system for measuring the velocity of nerve impulses originating outside the two pairs, as one need only measure the distance along the time base between the two sharp intersections (Fig. 1*a*).

A slight correction has to be made if the electrode pairs are much less than the total length of an impulse apart, but the complete data for a velocity determination are obtained from a single record of a single nerve excitation and there is no need to measure anything except distances along the base-line.

If one wishes to have a still more direct measurement of impulse velocity which is suitable for quite short lengths of nerve and which will sort out individual fibre groups in a mixed nerve, an electrical differentiator need only be added to the previously described system. In this case the (first) intersection is much sharper and corresponds not to the peak of the monophasic action potential but to the moment of steepest rise, which indicates the instant at which the nerve membrane becomes physiologically active at the electrode pair (Fig. 1*b*).

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The oral activity of certain androgens, oestrogens and augmenting substances. By C. W. Emmens. (*From the National Institute for Medical Research, Hampstead, London*)

Table I shows the approximate amounts of various androgens required to produce an average increase of 5 mm. in length plus height in the combs of a group of five Brown Leghorn capons, when administered in three daily doses in oily solution by (*a*) intramuscular injection, (*b*) unction on to the comb, or (*c*) mouth. The relative activity of the compounds is different by each method, consequently the ratio of the actual amount needed by mouth to that needed by injection is different for each androgen.

Table I. Approximate amounts (μg.) of various androgens needed to produce a 5 mm. increase in length plus height of the combs of Brown Leghorn capons, when given by different routes

Substance	Assay method			Dose by mouth/ Dose by injection
	Unction	Injection	By mouth	
Androsterone	3·0	500	4,500	9
Testosterone	2·1	85	2,500	30
Androstanediol	2·4	165	25,000	150
trans-Dehydroandrosterone	6·0	1500	22,500	15

Table II gives a similar comparison for certain oestrogens, and for the oral activity of ethinyl oestradiol, diethylstilboestrol, and the synthetic hexadiene compound described by Dodds, Golberg, Lawson & Robinson [1938 *a*, *b*]. The test used was the production of vaginal

TABLE II. Approximate amounts (µg.) of various oestrogens needed to produce 50 % positive responses in groups of ovariectomized mice

	Assay method		
Substance	Injection	By mouth	Dose by mouth/ Dose by injection
Oestrone	0·1	1·0	10
Oestriol*	1·1	3·0	3
Oestradiol	0·025	1·1	45
Ethinyl oestradiol	ca. 0·02	1·2	ca. 60
Diethylstilboestrol	ca. 0·1	0·4	ca. 4
Hexadiene compound	>0·1	<0·1	<1

* This sample of oestriol was more active by injection than another sample examined and may have contained a small amount of oestrone.

cornification in groups of twenty ovariectomized mice following two daily doses. Solution in oil was used for injection, and in propylene glycol for administration by mouth. The ratio of oral and subcutaneous doses for oestrone and oestradiol shown in the table agree with those found by David, de Jongh & Laqueur [1935] for mice (10 : 1 and 40 : 1 respectively).

Two materials which augment the effect of oestriol when injected with it in oily solution [Emmens, 1939] have also been investigated. The first of these, benzoic acid, is effective in a dose of 10 mg. in increasing sixfold the assayed potency of oestriol injected in two daily doses. It is, however, ineffective if given by mouth at the same time as oestriol is injected. The second material, the phenolic fraction of an extract of normal female urine [Callow, 1936], although itself of insignificant oestrogenic activity in the doses given, is capable of increasing approximately tenfold the assayed potency of oestriol. (Compare Freud, Dingemanse & Polak [1937] for similar results with androgens.) When given by mouth at the same time as oestriol is injected it can still produce a five or sixfold augmentation of the hormone. Such oral activity is indicative of a mechanism of augmentation not depending on delaying the absorption of active material from the subcutaneous tissues.

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Evidence of fat/fatty acid partition in human fat absorption.

By A. C. FRAZER and H. C. STEWART. (*From the Physiology Department, St Mary's Hospital Medical School, W.* 2)

Physical, chemical and physiological investigations [Frazer & Stewart, 1937*a*, 1938; Cunningham & Peters, 1938] show that at least the bright particles, seen in normal human serum under dark-ground illumination, are fat. Counts of these bright particles are used as a means of serial investigation of variations in blood fat. A rise in the particle curve always follows ingestion of neutral fat, and this represents the passage of fat through the blood stream on the way from the thoracic duct to the fat depots. We have shown [Frazer & Stewart, 1937*b*; Frazer, 1938*b*] that if equivalent amounts of fatty acid and glycerol are ingested there is no increase of chylomicrons although faecal analyses show that the fatty material is absorbed. This fatty acid presumably passes by the alternative route, the portal vein, to the liver—a supposition which is borne out by simultaneous portal and systemic counts in rats and also by feeding experiments with stained fat or fatty acid. These findings are difficult to reconcile with the current view of fat absorption [Verzar, 1936] according to which the importance of lipase lies in its being an essential in the preparation of the fat molecule for absorption. We have, therefore, put forward the fat/fatty acid partition hypothesis [Frazer, 1938*a*] which explains our findings and correlates them with those of the majority of other workers.

The object of this communication is to put forward evidence of fat/fatty acid partition in the human subject:

(1) If olive oil is taken by duodenal tube, a marked increase in chylomicrons very soon occurs. If an equivalent amount of oleic acid and glycerol is taken, no rise is found in the particle curve [Frazer, 1938*a*].

(2) Using a standard meal consisting of 2 oz. toast, 1 oz. butter and a large cup of tea, the effect of addition of lipase to the diet has been studied. Fig. 1 shows the particle curves obtained with an identical amount of fat in two subjects with and without added lipase. The continuous lines are the curves obtained with the standard meal and the interrupted lines show the effect of adding six grains of holadin to the meal.

Thus addition of lipase causes a very marked decrease in the systemic lipaemia which follows ingestion of fat as judged by these particle curves. This is surprising if lipolysis is an essential preliminary to absorption, but it would be expected if fat/fatty acid partition occurs. Thus, lipolysis may not be essential for fat to be absorbed, but an important function of lipase may be to determine what proportion of ingested fat is to pass direct to the liver for immediate metabolic use and how much is to pass unhydrolysed by the lymphatic pathway to be stored in the fat depots for the future.

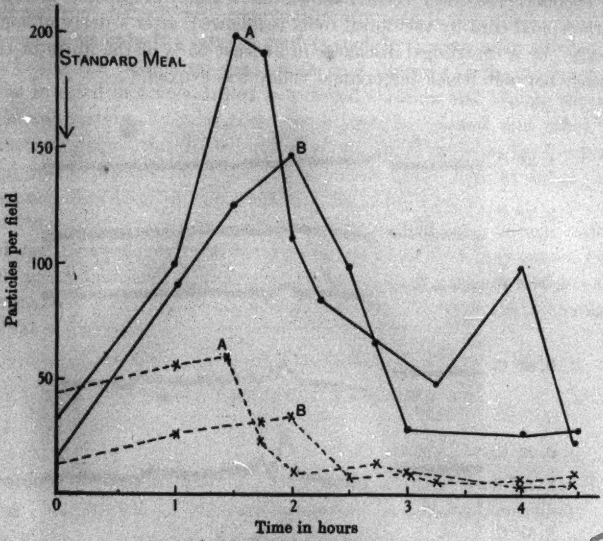

Fig. 1. Particle curves from two subjects A and B with and without lipase added to the standard meal. Full description in the text.

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Dorsal root reflexes. By DONALD H. BARRON and BRYAN H. C. MATTHEWS. (*From the Physiological Laboratory, Cambridge*)

Toennies [1938] has recently shown that a volley of impulses arriving at the spinal cord by the dorsal roots is followed, after a delay of some 4 msec., by a centrifugal discharge in at least 35 % of the fibres of the sensory nerve in which the original volley was evoked.

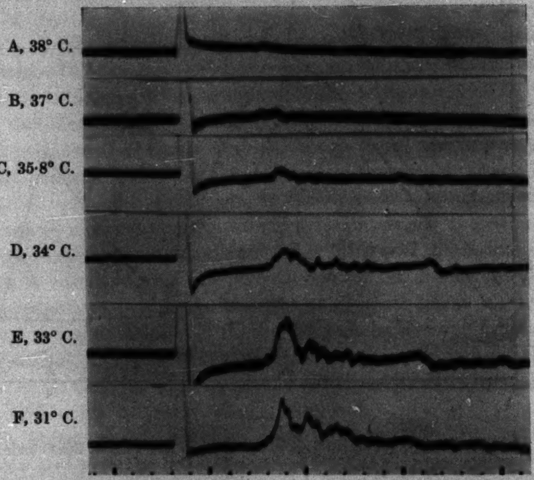

Fig. 1. Recording and stimulating electrodes on the saphenous nerve of a decerebrate cat just below the knee. Stimulus a single maximal break shock from a coreless induction coil. Temperatures by a lumbar puncture thermocouple. Time in F, 0·002 sec. intervals. 1 cm. = 1·5 mV.

In earlier work on the impulses leaving the cord by the dorsal roots we had seen nothing exactly comparable except in cats in a semi-convulsive state or under strychninization; we have therefore repeated some of Toennies' experiments on cats. We are able to reproduce effects of the same magnitude as those he described only if the spinal cord is cooled some 4–5° C. below its normal temperature.

In Fig. 1 E is shown the volley recorded from the saphenous nerve set up by a single break shock followed by the "reflex" discharge in a large fraction of the fibres present. The temperature of the cord recorded by a lumbar puncture thermocouple was 33° C. The records, Fig. 1 A–E, are a series taken on the same animal at various temperatures; at 38° C. (which we find to be the average temperature of the spinal fluid before operation) the effect becomes vanishingly small. These changes in the "dorsal root reflex" with temperature are seen if the whole animal is cooled, or if only the lumbar cord is cooled; this we have effected by perfusing the extradural space with cool Ringer's fluid entering by a tube inserted in a hole drilled in a dorsal vertebra and leaving through a sacral vertebra. Under these conditions the "dorsal root reflex" is practically absent at 38° C., but after the spinal cord has been exposed and roots dissected it is present in a few fibres even at 38° C., and becomes very large on superficial cooling of the cord.

From our experiments we conclude that in the normal animal "dorsal root reflexes" will occur in so few fibres that they cannot have any great functional significance. However, their presence in the cooled animal is of great interest because of the light it throws on the mechanisms of the spinal cord.

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Dorsal root potentials. By Donald H. Barron and Bryan H. C. Matthews. (*From the Physiological Laboratory, Cambridge*)

A volley of impulses arriving in the spinal cord via the dorsal root sets up a "reflex" discharge of impulses [Toennies, 1938] which pass peripherally in a certain proportion of the fibres of the dorsal roots. But cooling of the cord appears to be a necessary condition for it to appear in many fibres [Barron & Matthews, 1938*b*]. The phenomena concerned with the central production of the antidromic impulses have been investigated in decerebrate cats by observing the relationship between the slow potential changes in the dorsal roots and the occurrence of the dorsal root "reflex" discharges. We have shown [1938*a*] that the arrival of impulses in the cord via the dorsal root is followed by a slow central negativity which spreads peripherally by electrotonus. This slow potential change is accompanied, if the temperature of the spinal cord is slightly below normal, by impulses which are conducted peripherally. These impulses appear to be invariably related to the rising phase of the slow potential change in the same way that the impulse discharges of motor neurones are related to the slow potential changes in the ventral roots. This is illustrated in Fig. 1. We have previously suggested that the slow potential change of the dorsal root indicates the process responsible for the excitation of the secondary neurones; when the spinal cord is cooled, it appears that the same process may excite the primary sensory neurones owing, perhaps, to a slowing of accommodation at their central terminations.

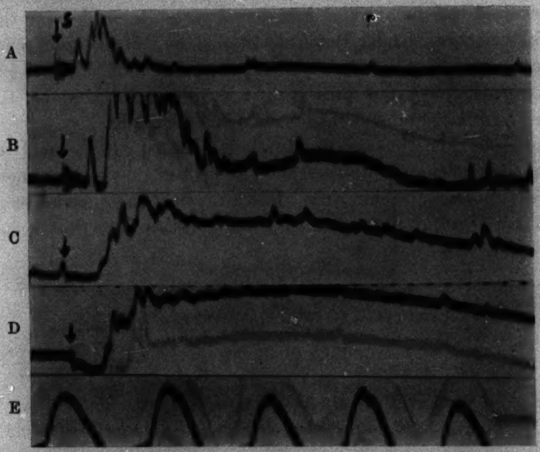

Fig. 1. Potentials recorded from dorsal rootlets in decerebrate cats. Stimulus S a single break shock to another dorsal rootlet. A. Dorsal root "reflex" recorded from a dorsal root starting 3·5 msec. after the stimulus, it is preceded by 2 msec. by impulses in recurrent fibres. Temp. 38° C. Total conduction distance in roots approx. 40 mm. B. Ditto, cord cooled. Sensitivity ×2 that of A. C and D. Ditto, recorded with proximal electrode 2 mm. from cord, showing slow dorsal root negativity and "reflex" occurring on its rising phase. Temp. 36° C. E. Time 100 d.v. per sec.

Further, we have observed that the dorsal root "reflex" discharges only occur after a nearly synchronous centripetal volley, such as follows a sharp tap on the foot or an electrical stimulus to a peripheral sensory nerve. A steady stimulus, however (e.g. pressure on the foot), fails to evoke a "reflex" discharge via the dorsal root. This may be due to the fact that it does not produce an abruptly rising slow potential centrally.

This suggestion, that the rise of the dorsal root negativity causes the dorsal root "reflex", agrees with Toennies' finding of summation under the conditions in which we have found that there is summation of the dorsal root potential to produce a steeper rise, and reduction of the dorsal root "reflex" under conditions such that the rise is reduced by the potential having already developed in response to a previous volley.

The effects of cooling the spinal cord are not, however, confined to the sensory side, de Almeida [1936] has shown that cooling of the frog's spinal cord induces convulsions. Similarly, we find that if the cord of a decerebrate cat is cooled below 31–32° C., spontaneous twitching, and irradiation of any stimulus occurs, much as when the animal is given strychnine. At each muscular twitch impulses are discharged not only along motor fibres but via many sensory fibres as well. It appears that one-way conduction at the synapse is so delicately balanced that even a fall of temperature of a few degrees causes a partial failure.

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